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CANCER RESEARCH

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Some Investigations on Cell Behavior under Various Conditions: *A Review**

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Some of the basic problems of cancer research arise in the field which we call cytobiochemistry. Besides fundamental research on the chemistry of cell behavior, we have been especially interested in the search for substances with tumor-inhibiting properties. This review is a survey of work on mitotic poisons, their antagonists and synergists, the chemistry of cell division, the relation between cell metabolism, growth, and division, and the behavior of components of tumor cells in transplantation.

1. MITOTIC POISONS

In 1937, Dustin (11) described the ability of colchicine to inhibit mitosis of cells (normal and malignant) *in vivo* (12, 13). Colchicine is one of the compounds that Dustin called "poisons caryoclasiques." In 1939, Ludford (82) described a similar action by several compounds on cell division *in vitro;* he called these substances "mitotic poisons." We found that not all the substances which Dustin called "poisons caryoclasiques" produced an effect on cells *in vitro*—i.e., a direct action on the cell in division. Thus, we propose to call a substance a "mitotic poison" only if its direct action on the cell has been proved. Examples of such substances are colchicine, acriflavine, podophyllotoxin, auramine, and others.

Any type of dividing cell of animals or plants would be suitable for testing the effect of a substance on mitosis. We used two methods: tissue culture (including fibroblasts, carcinoma, normal epithelium and other tissues) and the mouse ascites tumor.

* Based on talks presented at the Gordon Research Conference, AAAS, on August 27, 1951, and at the University of Wisconsin on September 20, 1951.

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Tissue culture (24).—Several cultures of chicken mesenchymal or heart fibroblasts, 48 hours old, were divided into two portions. One half was explanted as a control culture in a medium consisting of 1 drop of chicken plasma, 1 drop of embryonic juice, and 1 drop of saline solution. The second part, serving as the experimental culture, was explanted in a medium containing the solution of the substance to be investigated instead of the pure saline solution. The final concentration of the added substance was one-third that of the added solution and is expressed in μg/ml. At the end of 24 hours, control and experimental cultures were compared. The effect of a mitotic poison is so drastic that the effect can be observed qualitatively in the living culture (Figs. 1 and 2). For quantitative estimation, the cultures were stained and the numbers of mitoses counted and morphological changes recorded. To investigate effects on the cell spindle, the staining method of Ehrlich-Biondi was used, and the stained preparation was observed under the phase contrast microscope (24) (Fig. 3). In the case of colchicine, 0.01 μg/ml can be detected by its action on dividing fibroblasts. Since 1939, more than 1,000 chemical compounds have been investigated by this method, involving about 350,000 cultures of fibroblasts (reviewed by Lettré, 31, 35). We used this method to estimate the distribution of colchicine in the body; extracts of different organs were tested for their effect on tissue cultures. By this method—comparable in its sensitivity to the method of isotopically labeled compounds—we found that colchicine is excreted chiefly by the liver via the bile into the intestine; there is no urinary excretion. Even when toxic dosages were administered, the brain contained no amount detectable by this method (75). In

unpublished experiments with Roeder (Göttingen), we found that colchicine derivatives introduced by means of iontophoresis into the brain of guinea pigs had no acute toxic effect; the colchicine derivative could be detected in the liver 10 minutes after the iontophoresis.

Motion pictures are an important aid in studying the action of mitotic poisons on cellular behavior. Films of our studies are available to universities (33, 34).

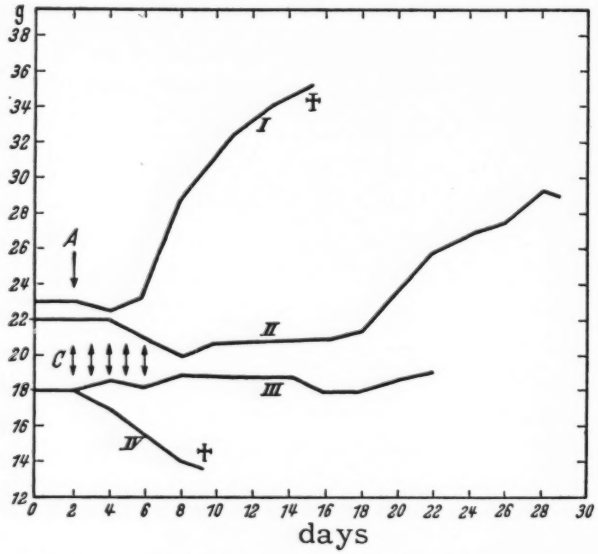


CHART 1.—Weight curves of mice with ascites tumor.
I—At *A*, 0.2 ml. tumor ascites inoculated intraperitoneally.
II—The same and at *C*, 10 μg. colchicine daily, as indicated.
III—No tumor inoculated; at *C*, 10 μg. colchicine daily, as indicated.
IV—No tumor inoculated; at *C*, 20 μg. colchicine daily, as indicated.
†—Death of mice.

The mouse ascites tumor.—In 1932 Loewenthal and Jahn (81) described a subline of the Ehrlich mouse carcinoma. By intraperitoneal injection of free cells of this tumor they succeeded in obtaining an ascites containing free tumor cells. Since 1940 we have used this ascites tumor for studying the influence of exogenous factors on its growth (25, 26, 36). The production of ascites, together with the increase in the number of tumor cells, drastically changes the weight curve of the mouse. The increase in weight may be 20–30 gm. Thus, the weight curve of the animal after the injection of the tumor cells is a good indicator of tumor growth. Following the injection of mitotic poisons, the tumor growth was inhibited, and the difference between the weight curves of the experimental animals and the control group can easily be observed (Chart 1). A comparison between the survival time of the control group and that of the treated group gives a further indication of the activity of a substance. In 1934 Collier and Jahn (7) mentioned the mitotic figures in the dividing tumor cells and called this tumor an excellent object for cytological experiments. Brodersen (4) described a morphological method for investigating the mode of action of a substance. Smears of ascites, taken at different times after the injection of a substance, were stained and the number of mitoses and morphological changes recorded. Brodersen thus was able to differentiate between the action of colchicine, acriflavine, and x-rays (Chart 2). We use this method routinely to compare chemical compounds. The effect of N-methylcolchicamide (NMC), a more effective derivative of colchicine, was demonstrated by this method (Fig. 4) (62). By fractionating the dosage of NMC, we could

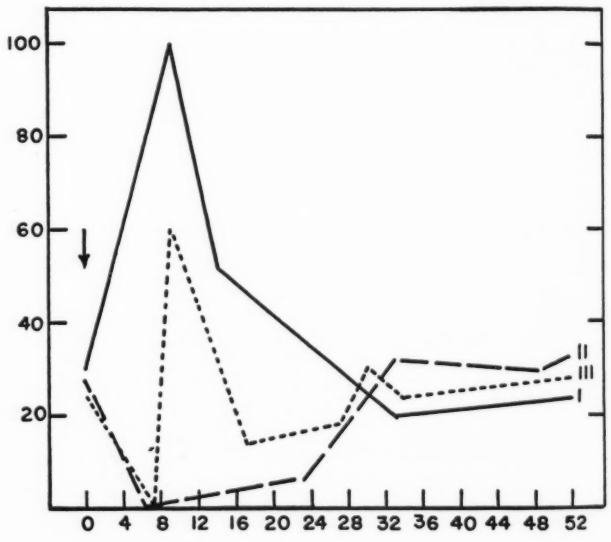


CHART 2.—Effects of different agents on the number of mitoses of the mouse ascites tumor.
I—Colchicine.
II—Acriflavine.
III—X-Rays.
Ordinate: mitoses per 1,000 cells.
Abscissa: hours after treatment.

show, for instance, that five injections of 3 μg. of NMC, given every 24 hours, are much more effective than a single injection of 15 μg. (Lettré and Bergdolt [55]).

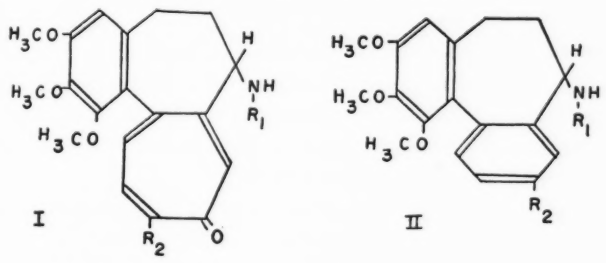
Following repeated injections of small doses of NMC and repeated transplantations of tumors treated in this way, a subline of the mouse ascites tumor was obtained which was resistant to this agent. We consider this resistant kind of tumor to be the result of a spontaneous mutation. This subline is also resistant to colchicine (61). Although cells of this resistant form divide by

mitosis, division cannot be influenced by colchicine or NMC. This finding is important for the theory of mitosis. It is important, too, for an understanding of the action of colchicine on the normal form of the ascites tumor. The objection has been raised that the inhibition of tumor growth was only secondary to a primary effect on the host. In investigations with the resistant form the hosts receive the same injury from the substance as in the case of the sensitive form. Therefore, the inhibition of the sensitive form is actually a direct effect on the tumor cell and not a secondary phenomenon.

Colchicine and derivatives.—The formula of colchicine (Table 1, I) proposed by Dewar (9, 10)

TABLE 1

COLCHICINE AND DERIVATIVES



FORMULA I

R_1	R_2	
$-CO-CH_3$	$-OCH_3$	colchicine
$-CO-CH_3$	$-OH$	colchiceine
$-H$	$-OH$	trimethylcolchiceinic acid
$-H$	$-OCH_3$	desacetylcolchicine
$-CO-CH_3$	$-OC_2H_5$	O-ethylcolchicine
$-CO-CH_3$	$-OC_3H_7$	O-propylcolchicine
$-CO-CH_3$	$-OC_4H_9$	O-butylcolchicine
$-CO-CH_3$	$-NH_2$	colchicamide
$-CO-CH_3$	$-NH-CH_3$	N-methylcolchicamide
$-CO-CH_3$	$-NH-C_2H_5$	N-ethylcolchicamide
$-CO-CH_3$	$-N(CH_3)_2$	N-dimethylcolchicamide

FORMULA II

$-CO-CH_3$	$-OH$	N-acetylcolchinol
$-CO-CH_3$	$-OCH_3$	N-acetylcolchinol methyl ether
$-H$	$-OH$	colchinol

in 1945 now seems to be well established. Colchicine, an alkaloid from *Colchicum autumnale*, contains in its molecule three rings: one aromatic ring with three methoxyl groups, one seven-membered ring with an acetylated primary amino group, and one tropolone ring with a methoxyl group. Since 1940, we have been investigating degradation products and derivatives of colchicine to clarify the relationship between chemical constitution and activity as mitotic poison (58) (Tables 1 and 2). The hydrolysis of colchicine leads to colchiceine, the methyl group (R_2 in Table 1) being split off. Colchiceine is still a mitotic poison, but quantitatively its activity is only $\frac{1}{100}$ of that of colchicine. Further hydrolysis removes the acetyl group. The resulting compound, trimethylcolchiceinic acid, has no activity as a mitotic poison (cf. Brues and Cohen [5]). Thus, we might conclude that the acetyl group is indispensable for activity. However, if we transform this compound into its methyl ether, desacetylcolchicine, we get a substance of almost the same activity as colchicine, although it does not contain the acetyl group. Trimethylcolchiceinic acid is an amphoteric system, and this seems to be the reason for its inactivity. These results, published in 1942 (29), are in agreement with the findings of Ullyot and his associates (14, 17). Windaus (93) transformed colchiceine to N-acetyliodocolchinol and acetylcolchinol; both substances are effective anti-

TABLE 2

ANTIMITOTIC ACTIVITY OF COLCHICINE DERIVATIVES ON CHICKEN HEART FIBROBLASTS

Derivative	Level showing activity (μg/ml)
colchicine	0.01
isocolchicine	0.4
colchiceine ethyl ether	0.04
isocolchiceine ethyl ether	9
colchiceine propyl ether (mixture)	0.8
colchiceine butyl ether (mixture)	2.5
colchiceine	4.5
desacetylcolchicine	0.05
trimethylcolchicinic acid	100 no activity
colchicinic acid	100 no activity
colchicamide	0.01
N-methylcolchicamide	0.0025
N-ethylcolchicamide	0.003
N-propylcolchicamide	0.08
N-butylcolchicamide	0.9
N-dimethylcolchicamide	0.005
N-methyl,N-propylcolchicamide	0.5
N-benzylcolchicamide	3
N-acetyliodocolchinol	0.4
N-acetylcolchinol	0.6
colchinol	0.1
N-methylcolchinol methyl ether	4
N-dimethylcolchinol methyl ether	5
hexahydrocolchicine	0.5
oxycolchicine	10
trimethoxyhomonaphthide	80 no activity
N-acetylcolchinic acid anhydride	60 no activity

mitotic agents. Colchinol (Table 1, II), obtained by hydrolysis of N-acetylcolchinol, is the simplest degradation product of colchicine that has activity. If the tropolone ring is destroyed by oxidation (N-acetylcolchinic acid anhydride) or if the nitrogen is removed from N-acetylcolchinol methyl ether to desaminocolchinol methyl ether or to the so-called trimethoxyhomonaphthide of Windaus, there is a complete loss of activity. Tropolone and its methyl ether do not show any antimitotic activity (71).

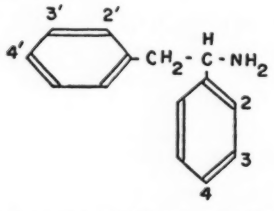
By remethylation of colchiceine with diazo-

methane, two substances result: colchicine and an isomeric substance, isocolchicine (Sorkin [88]). Isocolchicine is a derivative of an isomeric form in the tropolone ring. The activity of isocolchicine is $\frac{1}{60}$ that of colchicine. The ethyl ether of colchiceine, corresponding to colchicine, has $\frac{1}{5}$ the activity of colchicine, but the ethyl ether corresponding to isocolchicine only $\frac{1}{1000}$ (59).

All these compounds are less effective than colchicine. The reaction of colchicine with ammonia produces colchicamide, comparable in its activity on fibroblasts to colchicine. A great number of substituted colchicamides were obtained by the analogous reaction of colchicine with primary and secondary amines (28). The activity of N-methylcolchicamide (and the N-ethyl and N-dimethyl derivatives) is greater than that of colchicine. The derivatives bearing substituents with longer carbon chains on the nitrogen atom have less activity (Lettré [36, 47, 67]).

TABLE 3

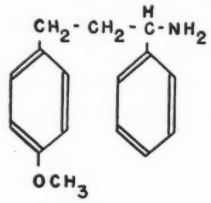
SOME EXAMPLES OF THE RELATION BETWEEN STRUCTURE AND ANTIMITOTIC ACTIVITY IN THE STILBYLAMINE SERIES



III. α,β-Diphenylethylamine ("Stilbylamine")

Substituents	Level showing activity (μg/ml)
unsubstituted	none
4'-methoxy-	4–5
3'-methoxy-	20
2'-methoxy-	none
4'-ethoxy-	0.4
4'-propoxy-	0.8
4'-butoxy-	1.8
3',4'-dimethoxy-	none
4,4'-dimethoxy-	"
4-methoxy-	"
3',4'-methylendioxy-	6
3',4'-dimethylendioxy-	4
3',4'-tetramethylendioxy-	none
3',4'-hexamethylendioxy-	"
3',4',5'-trimethoxy-	"
4'-methyl-	10
3',4'-trimethylen-	10
3',4'-tetramethylen-	10
2',3'-benz-	none
3',4'-benz-	"
3,4,3',4'-tetramethoxy-	"
3,4,3',4'-(methylen-dioxy)-	"

Synthetic analogs of colchicine.—In 1940, we started to synthesize substances similar in their structure to colchicine. At this time, the Windaus formula of colchicine (93) was still accepted. Cook and Engel (8) made open-ring analogs of compounds corresponding to this suggested formula of colchicine. In applying their principles to the Windaus formula, Lettré and Fernholz synthesized 4'-methoxy-α,β-diphenylethylamine (4'-methoxy-stilbylamine) (Table 3) (58) and found it active on fibroblasts at 4 μg/ml. α-Phenyl-γ(*p*-methoxyphenyl)-propylamine (compound IV),

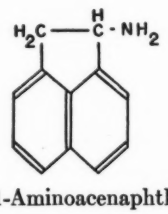


IV. α-Phenyl-γ-(*p*-methoxyphenyl) propylamine

analogous to the new colchicine formula, did not show any antimitotic activity. Therefore, we thought that the Windaus formula for colchicine was correct with respect to the size of the B-ring and the position of the nitrogen.

We investigated a great number of derivatives of α,β-diphenylethylamine ("stilbylamine") and found a number of active compounds (Table 3) (56, 57, 58). The 4'-ethoxy derivative is the most active of these synthetic compounds; it was separated into its optically active forms (77). Only the isomer with (−)-rotation proved to be effective; the (+)-isomer has no or only $\frac{1}{100}$ of the activity of the (−)-isomer. Hence, its activity as a mitotic poison is stereochemically specific.

The simplest synthetic compound with an antimitotic activity on fibroblasts is 1-aminoacenaphthene (V), $C_{12}H_{11}N$. However, compared to



V. 1-Aminoacenaphthene

colchicine, $C_{22}H_{25}O_6N$, its activity amounts to only $\frac{1}{10,000}$ (79).

Alkaloids.—It seemed of interest to determine whether other alkaloids would have an effect on cell division similar to that of colchicine. We tested 83 alkaloids and derivatives for their action on fibroblasts (50, 51). These alkaloids are listed in two groups (Tables 4 and 5): the first group has no chemical relation to the stilbylamine group, the second group are alkaloids which contain the

stilbylamine group. Among the first group there was no mitotic poison; but in the second group we found several acting as mitotic poisons: narcotine, gnoscopine, chelidonine, homochelidonine, methoxychelidonine, and protopine. It is striking that

TABLE 4

ALKALOIDS NOT RELATED TO STILBYLAMINE
(Not mitotic poisons)

tyramine	lupinine	eseridine
hordenine	lupinidine	aconitine
ephedrine	cinchonine	strychnine
mescaline	cinchonidine	brucine
hygrine	quinine	emetine
nicotine	quinidine	cephaeline
coniine	cinchotenine	ergotamine
lobeline	pellotine	lycopodine
piperine	anhalonine	lycoctonine
atropine	carnegine	agaricine
hyoscyamine	cotarnine	aricine
scopolamine	yohimbine	gelseminine
ecgonine	aspidospermine	taxine
cocaine	piloacarpine	veratrine
pelletierine	physostigmine	solanine

TABLE 5

ALKALOIDS RELATED TO STILBYLAMINE
(Mitotic poisons in italics)

I. aporphine group:
apomorphine
morphothebaine
corytuberine
corydine
bulbocapnine
boldine
glaucine
laurotetanine
laurotetanine methyl ether
N-acetyllaurotetanine

II. papaverine group:
papaverine
laudanosine
pavine
laudanine
laudanosoline
narcotine
gnoscopine
narceine
hydrastine
morphine
codeine
oxyacanthine
rhoeadine
d-tubocurarine

III. berberine group:
berberine
tetrahydroberberine
16,17-dihydrodesoxyberberine
palmatine
tetrahydropalmatine
jatrorrhizine
tetrahydrojatrorrhizine
tetrahydrocoptisine

IV. chelidonine group:
chelidonine
homochelidonine
methoxychelidonine
chelerythrine
sanguinarine

V. cryptopine group:
protopine
cryptopine

the active alkaloids contain the stilbylamine group. It was mentioned before that this structural element seems to be essential for antimitotic activity in the synthetic compounds. The type of substitution is of importance for activity, and this may explain why not all alkaloids with the stilbylamine group are active.

Quinones.—The inhibiting effect of quinones on cell division was discovered by E. F. Lehmann (23). For his experiments he used eggs of Tubifex (a species of ringworm which lays eggs during the whole year and thus is on hand for experiments at any time). Lehmann found that benzoquinone naphthoquinone and phenanthrenequinone had an extraordinarily strong effect, even surpassing that of colchicine on these eggs (Table 6). We may visualize the mechanism of action of these quinones to be a reaction with SH-groups, since *p*-benzoqui-

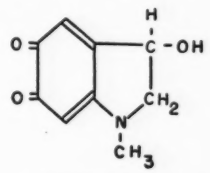
TABLE 6

ANTIMITOTIC ACTIVITY OF SOME QUINONES, COMPARED TO THAT OF STILBESTROL AND COLCHICINE

Antimitotic substance	Minimal active dose (for Tubifex eggs)
stilbestrol	1:300,000
colchicine	1:30,000
benzoquinone	1:2,000,000
naphthoquinone	1:900,000
phenanthrenequinone	1:30,000,000

none can add cysteine. Thus, the effect of quinones would be the consequence of SH-group blocking.

The investigation on the effect of epinephrine on tissue cultures resulted from a study of the mitotic poisons of the stilbylamine type which contain the sympathicomimetic phenylethylamine group. Numerous derivatives of this group, such as mescaline, tyramine, hordenine and others, proved to be inactive (49). Only epinephrine showed a detectable activity with a dose of 100 μg/ml. After the addition of epinephrine, the tissue culture medium became red-brown, so that we may assume that an oxidative product was formed which had antimitotic activity. Thus, we can define epinephrine as a promitotic poison. When at the same time either vitamin C or glutathione was added, oxidation of epinephrine was inhibited and, in consequence, its effect on mitosis. For a long time we have searched for an oxidative product which in its constitution is related to stilbylamine. In 1947 however we found that pure adrenochrome (VI) has this effect (74). According



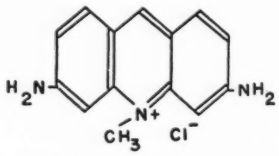
VI. Adrenochrome

to this, we suggest that the active product of epinephrine is effective on cell division through its quinonoid character.

Organometallic compounds.—The discovery of the effect of mitotic poisoning by organometallic compounds was stimulated by A. Klages (21). He observed that organic compounds of Hg which were being tested for antimycotic activity induced polyploidy in the treated seeds. At the beginning of 1943, we tested the effect of organic compounds

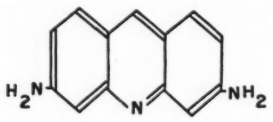
of mercury on chicken heart fibroblasts and observed an inhibiting effect on mitosis. Many compounds of the type Hg-R-X were effective, R being an aliphatic or aromatic organic radical and X, an anion. Inorganic salts of Hg such as $HgCl_2$ and $Hg(CN)_2$, as well as compounds of Hg completely substituted with organic radicals like Hg-diphenyl, proved to be ineffective. Similar behavior has been observed with other metals, e.g., lead, bismuth, tin, arsenic, and antimony, which also show antimitotic activity only when in mixed organic-inorganic compounds (31, 32, 35).

Mitotic poisons of the acriflavine type.—Acriflavine (VII) is the methochloride of 3,6-diamino-



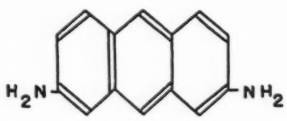
VII. Acriflavine

acridine. Its effect can be demonstrated on both fibroblasts (6) and mouse ascites tumor (27). Our first investigations dealt with the question which of the substituents in the molecule are necessary for the effect (31, 32, 35). Proflavine (VIII), or



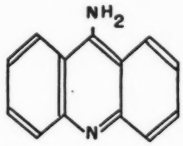
VIII. Proflavine

3,6-diaminoacridine, had the same effect, qualitatively as well as quantitatively. Acridine and acridine methochloride, however, were ineffective. Thus, the amino groups in the acridine skeleton appeared to be indispensable for activity. We then tested whether a molecule similar in structure to proflavine and with the same distance between the amino groups would show the same effect. 2,7-Diaminoanthracene (IX) is such a compound.



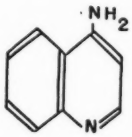
IX. 2,7-Diaminoanthracene

Even in high dosages it showed no inhibitory effect on cell division, nor did 2,7-diaminocarbazole. Likewise, "open" molecules built up in analogy to proflavine, as such *p*-diaminodiphenylmethene and *m*-diaminodiphenylamine, were ineffective. Hence, analogy to the form of the molecule of proflavine was not essential for activity. We obtained further information from the investigations on monoaminoacridines. Among the tested 3-, 4-, and 9-aminoacridines, only the 3- and 9-derivatives (X)



X. 9-Aminoacridine

proved to be effective, the 4-amino derivative showing no effect. Quantitatively, the activity was considerably lessened. For chicken heart fibroblasts 100 μg/ml were needed, whereas proflavine was effective at 2 μg/ml. We found simpler compounds related to 9-aminoacridine which were also effective. The condensed aromatic ring system may be omitted without loss of activity. 4-Amino-quinoline (XI) and 4-aminopyridine



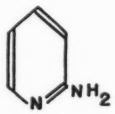
XI. 4-Aminoquinoline

(XII) showed an inhibiting effect on cell division

XII. 4-Aminopyridine

as well.

4-Aminopyridine was the simplest active molecule which we obtained in the acriflavine series. According to our former investigations pyridine itself showed no inhibiting effect on cell division. A comparison of the three isomeric aminopyridines proved that the 2- (XIII) and 4-aminopyridines



XIII. 2-Aminopyridine

were effective, whereas even high dosages of 3-aminopyridine were ineffective. The inactive 3-aminopyridine differed chemically from the other two isomeric products, inasmuch as a tautomeric form of it does not exist, whereas the effective isomers were able to form products of the diimine type. Likewise, the active aminoacridines and aminoquinolines could form tautomeric products, while the inactive members of these series could not. The inactivity of aromatic amines

of similar structure bore out this conclusion. Thus, we may summarize the results as follows: aminoacridines, aminoquinolines, and aminopyridines produce an inhibiting effect on cell division provided that they may form tautomeric products of the diimine type.

If the mitotic poisons of the acriflavine type act as antagonists of the purines and pyrimidines in the nucleic acids, all chemical analogs of purines and pyrimidines ought to be mitotic poisons of the acriflavine type. Experiments do not confirm this hypothesis. However, we are able to increase the activity of the basic types of aminopyridines by substitution, producing a closer analogy to the natural pyrimidines. 6-Methoxy-2-aminopyridine, for instance, is more effective than 2-aminopyridine itself, possibly since the methoxy derivative is more similar to cytosine. However, a number of synthetic analogs, e.g., aminothiazoles, aminoquinazolines, and aminotriazines, produced no effect on chicken heart fibroblasts.

Hormones.—As mentioned before, epinephrine acts as an antimitotic agent after its transformation into adrenochrome. We tested the effect of a great number of hormones on cell division of fibroblasts. Only estradiol, in the form of the sodium salt of its phosphoric ester, had an inhibitory effect at a dosage of 40 μg/ml. Stilbestrol also produced a very pronounced effect (30). Since hormones are regulators of metabolic processes, we must assume that the processes of cell division are also regulated by hormones and that hormonal specificity exists in the regulation of different types of tissues.

Although adrenochrome inhibits mitosis of fibroblasts, it has no effect on carcinoma cells *in vitro*. This forms the basis of our hypothesis (29, 49) that malignancy results from a type of mutation of a cell, as a result of which hormones regulating the normal type are no longer effective against the malignant type. Although the regulators are present, they are ineffective because the cell has changed. The alterations of cell metabolism alone would not explain the capacity of a malignant cell for unlimited growth under conditions where a normal cell is not able to grow. There must exist a disturbance of the regulating system between cell and host.

2. Antagonists and Synergists of Mitotic Poisons

For the most important types of mitotic poisons we are able to determine the point of attack inside the cell during mitosis. The determination of the mechanism of action is based on (*a*) the morphological picture of the treated cell and (*b*) the discovery of factors able to act in an antagonistic or synergistic way.

From the morphological point of view, acriflavine represents the classical type of a chromosomal poison, according to Bauch (2) and to Bucher (6). It produces sticking and clumping (pyknosis) of chromosomes. Chemically, the effect of acriflavine is attributable to the formation of a complex with the nucleic acids. We were able to counteract the effect of acriflavine on fibroblasts *in vitro* by adding nucleic acid to the culture medium (either ribo- or desoxyribonucleic acid) (64). These results are similar to those of McIlwain (83) with bacteria. Brodersen (4) showed that the injection of acriflavine decreased the number of mitoses in mouse ascites tumor, thus demonstrating its influence on nucleic acid metabolism. The reaction with the chromosomes is easily shown morphologically, but a reaction with the ribonucleic acid of the mitochondria can be proved just as easily: enzymatic reactions of the mitochondria can be inhibited by acriflavine. It seems important that during the time of the minimum of mitosis in the ascites tumor we find a maximum of amitotic divisions (78). We can summarize the action of acriflavine as follows: the nucleotides and the nucleic acids of the cell (both those of the nucleus and those of the cytoplasm) react with acriflavine, thus inducing a disturbance of either the polymerization or the reactions of the nucleic acids. The morphological aspect is modified by the dosage and by the stage of the cell at the moment of reaction.

The antimitotic action of organometallic compounds could be counteracted by the addition of cysteine or other compounds containing SH-groups (65). Thus, we consider their mode of action to be a reaction with SH-groups of cell components which are functionally important during mitosis. There are many enzymes the activity of which depends on their number of free SH-groups and which are important for cell metabolism but not directly for cell division. According to L. Rapkine (85), a maximum of free SH-groups occurs just before cell division. Thus, at that moment the organometallic compounds can react with certain SH-compounds important for cell division. Godeaux (15) was the first to show that the contractibility of actomyosin is inhibited by poisoning SH-groups; the antimitotic activity thus may be interpreted as the inhibition of the contractibility of cell components indispensable for mitosis.

The mode of action of colchicine was elucidated by synergistic compounds rather than by antagonists. We found a great number of sub-

stances which increase the activity of colchicine on fibroblasts in tissue culture (44, 45, 63, 66–73). These substances belong to different chemical groups. These substances can be divided into two groups: the members of the first group only show a synergistic effect when a threshold or overthreshold dosage of colchicine is used, the members of the second can activate even an underthreshold dosage of colchicine. As an example of the first group, data on bulbocapnine are presented in Table 7, and, as an example of the second group,

TABLE 7

SYNERGISTIC EFFECT OF BULBOCAPNINE ON COLCHICINE

Dosage μg/ml		Percentage of mitoses
Colchicine	Bulbocapnine	
	10	1.5
	20	2.2
	40	1.8
	80	2.8
0.005	8	1.1
0.0055	8	1.7
0.006	8	2.3
0.008	8	24.4
0.01	5	23.8
0.02	8	46.6

those concerning phlorhizin in Table 8. Bulbocapnine acts as a synergist only with a dosage of colchicine which is high enough to be effective alone, but phlorhizin can produce an effect with a dosage of colchicine too low to be effective by itself. These substances and the other synergists have no effect alone as mitotic poisons. Besides bulbocapnine, the alkaloids thebaine, glaucine, and laurotetanine are also highly effective synergists (Table 9).

TABLE 8

SYNERGISTIC EFFECT OF PHLORHIZIN ON COLCHICINE

Dosage μg/ml		Percentage of mitoses
Colchicine	Phlorhizin	
		1.9
	375	2.0
	188	2.0
	94	1.8
	47	1.6
0.04		54.6
0.02		27.2
0.011		6.4
0.01		5.5
0.008		3.5
0.0055		2.2
0.0045		2.6
0.003		2.4
0.011	188	80.0
0.011	94	47.2
0.011	6	16.9
0.011	3	12.3
0.011	1.5	6.6
0.0055	94	13.7
0.0045	94	5.3
0.003	94	4.6

The behavior of the steroid hormones is similar to that of the alkaloids. Most steroid hormones have a synergistic effect; again, there are two types: one increasing the activity of a threshold dosage of colchicine, the other able to activate an underthreshold dosage. Testosterone, for instance, reinforces the activity of the threshold dosage of 0.01 μg/ml, while estrone is without any effect at this level of colchicine. However, an overthreshold dosage of 0.016 μg/ml colchicine can be activated by estrone (Table 10). Adrenal cortical hormones, both desoxycorticosterone and cortisone, are pronounced synergists of colchicine. Unlike desoxycorticosterone, cortisone is able to make effective an underthreshold dosage of colchicine (Table 10). Phosphocreatine can counteract the syner-

TABLE 9

SYNERGISTS OF COLCHICINE

bulbocapnine	+	papaverine	+
corydine	+	tetrahydro-papaverine	+
glaucine	+		
laurotetanine	+	berberine	+
laurotetanine methyl ether	+	tetrahydro-berberine	+
apomorphine	−	morphine	−
morphothebaine	−	codeine	(+)
corytuberine	−	thebaine	+
boldine	−	*d*-tubocurarine	−(+)
N-acetyllauro-tetanine	−	quinine	+
		strychnine	+
chelerythrine	+	veratrine	+

\+ = synergistic.
− = not synergistic.

TABLE 10

SYNERGISTIC EFFECTS IN TISSUE CULTURES OF STEROID HORMONES ON COLCHICINE

Hormone*	Colchicine (μg/ml)	Percentage of mitoses
	0.01	5.0
testosterone		2.4
testosterone	0.01	21.0
testosterone propionate		2.6
testosterone propionate	0.01	13.0
estrone		2.6
estrone	0.01	3.4
	0.016	14.2
estrone	0.016	21.3
	0.024	42.5
estrone	0.024	62.2
	0.005	2.0
	0.0075	2.5
	0.010	5.5
desoxycorticosterone acetate	0.005	1.2
desoxycorticosterone acetate	0.0075	2.0
desoxycorticosterone acetate	0.010	9.0
cortisone	0.005	3.3
cortisone	0.0075	8.2
cortisone	0.010	31.3

* Hormones added in solid form to the medium of the tissue culture.

gistic effect of desoxycorticosterone, but not that of cortisone.

These results can be interpreted by assuming that colchicine is active in some way in the system of contractile elements during cell division. The contraction of the cell spindle and the pinching in of the cell during cell division are thus analogous to muscular contraction. It is remarkable that this analogy has been discussed since the end of the last century by morphologists (see Heidenhain [18]), but it was not until 1947 that the chemical analogy was discussed by Brachet (3). He proposed the hypothesis that adenosine triphosphate (ATP) is the source of energy for spindle contraction as well as for muscle contraction. According to Szent-Györgyi (90), actomyosin, isolated from muscle and precipitated in the form of threads, can be induced to contract by the addition of ATP. The metabolism of the cell or the muscle can produce the ATP needed for contraction either from phosphocreatine or from the degradation of carbohydrates by glycolysis or respiration. Colchicine, however, inhibits respiration or glycolysis only in amounts a thousand-fold higher than the amount necessary for inhibiting mitosis. Therefore, its effect on mitosis cannot be explained by its direct effect on metabolism. The same statement can be made concerning phosphatases. ATPase of liver or muscle is inhibited only by extremely high amounts of colchicine (47, 52). We found, however, that ATP added to the tissue culture medium counteracts the action of colchicine for some hours—as long as ATP is stable under these conditions (52). Bárány and Palis (1) showed that colchicine influences the decrease in viscosity of a mixture of actomyosin and ATP. It seems important that the effective dosages used by these authors are comparable to those which inhibit mitosis. Thus, we may formulate the theory that colchicine inhibits a reaction between ATP and a contractile system of the actomyosin type inside the cell during mitosis. The activity of colchicine depends on the amount of ATP inside the cell: i.e., it is inversely proportional to the amount of ATP. On the other hand, its activity depends on the state of the contractile material.

From this point of view, we are able to interpret the synergistic effect of phlorhizin by its ability to poison phosphotransferases, thus decreasing the level of ATP inside the cell. Bulbocapnine has not previously been known to have an effect on the production of energy-rich phosphate bonds. It is a well known muscle poison which produces catalepsia; therefore, its synergistic effect may be considered an effect on the contractile proteins. The synergistic effect of veratrine, also a muscle poison, is antagonized by the addition of phosphocreatine.

These results suggest investigations concerning the role of steroid hormones in the metabolism and production of ATP. It is thought by us that steroid hormones have a "carrier function" in the processes of phosphorylation.

Contrary to Ludford (82), who interprets the effect of colchicine from the morphological point of view as an inhibition of spindle formation, our results suggest only a dysfunction of the spindle. A contractile system, a unit of form and function, may change its shape when its function is disturbed. Real suppression of spindle formation is only brought about by factors which are also able to inhibit blood clotting—e.g., heparin (19) or substances able to bind calcium.

Colchicine and N-ethylcolchicamide have been used for local treatment of skin cancer, papilloma, condyloma, and breast cancer by Brodersen (4). Colchicine has been applied in erythroplasia by Schönfeld (87). Hirsch (20) described cases of precancerous nevi and rodent ulcers which disappeared after treatment with a combination of bulbocapnine and colchicine. The great number of combinations of synergists with colchicine gives a broad field for investigations in cases of skin cancer.

3. Chemistry of Cell Division

We consider the study of mitotic poisons as a tool to investigate reacting components during mitosis, and we may assume that these components are also important in uninfluenced cell division. During interphase the metabolism of cytoplasm (i.e., respiration) predominates; in addition, protein synthesis occurs in both nucleus and cytoplasm. During early prophase, an increase in nuclear material occurs. During prophase the chromosomes consisting mainly of desoxyribonucleic acid become visible; chemically, this indicates formation and polymerization of nucleotides. The precipitation of nucleic acids in the form of chromosomes seems to be a condition *sine qua non* of the dissolution of the nuclear membrane. In the interphase nucleus, nucleotides and polynucleotides are attached to the nuclear membrane; in this stage, the membrane seems to have the character of a nucleoprotein stable against proteolytic enzymes. If the nucleotides are removed, the remaining membrane is susceptible to proteolytic activity. When this does not take place, the nuclear membrane cannot be dissolved, and the nucleus can only divide directly by amitosis. We consider the formation of the cell spindle to be a

process similar and related to that of blood clotting. Spindle contraction, leading to the separation of the chromosomes, is, as mentioned above, a process similar to muscle contraction. The process of pinching in of the cytoplasm is a result of the contractibility of the cell surface (43).

4. Cell Metabolism, Growth, and Cell Division

In 1926, Warburg (92) summarized his investigation on cell metabolism and growth in the statement: "No growth without glycolysis." He found that in malignant cells aerobic glycolysis is predominant over respiration, in embryonic cells it is comparable to respiration, but in adult normal cells glycolysis is less than respiration. Although not every tissue with predominance of glycolysis must be a growing one, there is some relation between growth and glycolysis. Growth is a two-step phenomenon: the first step is the preparation of a cell for division, and the second step is the division itself. Thus, the question arises whether glycolysis is related to the first or to the second step. The findings of O'Connor (84) on embryonic cells of different ages show that there exists a direct proportionality between the number of cell divisions and aerobic glycolysis. A possible explanation would be that during division glycolysis predominates, respiration being inhibited.

The results of the following experiments are in agreement with this finding (48, 53, 54). In a tissue culture of fibroblasts, there are on the average 98 per cent of cells in interphase and 2 per cent in the stage of division. During mitosis, the spindle-shaped cells become spherical. Cell division is characterized by movement of the cell surface, which pushes out pseudopodia. From the morphological point of view we must consider this stage of the moving cell as functionally connected with mitosis and the movement of chromosomes. But in motion pictures of cells under the action of mitotic poisons, we have seen that plasma motility occurs, although the separation of the chromosomes is inhibited (33). Furthermore, we were able to show that plasma motion can be induced by all factors inhibiting respiration but not effecting glycolysis. As the enzymes of respiration are mostly localized in the mitochondria, dyes with a more or less pronounced affinity for mitochondria prove to be highly effective—e.g., victoria blue, janus green, and others. Poisons of respiration such as potassium cyanide or the removal of oxygen have the same effect (reversible in the latter).

The link between metabolism and motion appears to be the effect of ATP on contractile proteins. Respiration produces at least 12 moles of ATP per mole of hexose but glycolysis only 2 moles of ATP. Thus, the change from respiration to glycolysis brings about a decrease in the ATP level of the cell. We interpret the induction of motion of the cell surface as a change from permanent contraction to local periodic contractions. This means that the surface of the "resting cell" is not in a real state of rest, but in a state of permanent contraction—a tonus, needing more chemical energy than the periodic contractions characteristic of mitosis. In consequence, the induction of cell motion by poisons of respiration should be compensated by the addition of ATP; this was confirmed experimentally. In agreement with Lewis (80), we consider the cell surface as a contractile layer and suggest that this layer has the qualities of actomyosin; the stage of contraction depends on the ATP level and thus on cell metabolism. It seems of interest that the same hypothesis has been discussed by Goldacre (16) with regard to the movement of amoeba. As shown above, the cell spindle needs ATP for its contraction. During mitosis, there are at least two contractile systems: (*a*) that of the cell surface, relaxed and needing only a small amount of ATP, and (*b*) that of the spindle and related contractile fibers needing ATP. The addition of ATP to a tissue culture of fibroblasts reinforces both systems. We would expect to find that the cell surface cannot relax after treatment with exogenous ATP, and, indeed, most of such cells in metaphases are not round but spindle-shaped, as are resting cells (Fig. 5). The separation of the chromosomes by contraction of the spindle fibers is favored, and cell division follows. From stained preparations it seems as if cell division in many of these cases takes place by tearing of the cells (Fig. 6); by direct observation of living fibroblasts with ATP added, however, we have not yet been able to observe this type of division. This type of cell division in an uninfluenced cell could only be produced by a metabolism with an extremely high production of ATP.

Together with the induction of plasma motion in resting cells by treatment with respiratory poisons, we observed the pinching off of plasma bubbles which then moved freely within the medium. It is believed that these moving lumps consist of a contractile layer and an enzyme system producing ATP sufficient for local contractions but not for permanent contraction. It is our opinion that this is the simplest biological system able to move (by means of carbohydrate metabolism), and we are engaged in attempts to combine actomyosin with enzyme systems, with the aim of "synthesizing" a primitive protoplasm-like system with motion and metabolism.

In general, cell shape and stability of cell shape

depend on the ATP level of the cell and thus on its metabolism. If metabolism produces a sufficient amount of ATP, permanent contraction of the surface is possible, and the cell shape is stable. A smaller amount of ATP results in periodic surface contractions, and the form of the cell is not stable. Such an unstable system not only may move but is forced to be in motion as long as the same metabolic conditions obtain: this is uncontrolled motion. On the other hand, metabolism with high production of ATP is able to control cellular motion.

As far as the cancer cell is concerned, we can conclude that the greater part of glycolysis is connected with the interphase; we consider this as related to synthetic processes (see Zamecnik *et al.* [94]). The mitosis of cancer cells is characterized by a short prophase and a long metaphase, while that of normal cells conversely has a long prophase and a short metaphase (Koller [22]). We interpret the short prophase as the result of an acceleration of synthetic processes in the cancer cell, the long metaphase as a result of a deficiency in ATP (37).

5. Behavior of Components of Tumor Cells in Transplantation

Since 1949, we have carried out experiments to determine whether components of tumor cells are able to induce tumors and whether reconstruction of a tumor cell out of its components is possible (38–42) (similar to Stasney *et al.* [89]). The simplest method to get a complete mixture of all components is homogenization. We used a blendor at a speed of 18,000 r.p.m. The mouse ascites tumor, the Walker carcinoma, and the Jensen sarcoma of the rat lost their ability to produce tumors when they were homogenized sufficiently for complete cell destruction. With the Walker carcinoma, these findings are in agreement with those of Tourtelotte and Storer (91). From these results, we can conclude that the reconstruction of an intact cell out of its components is impossible, at least for the types of mammalian tumors investigated and the conditions employed. With the Rous sarcoma, however, we were able to produce tumors with the homogenate. The virus is not destroyed mechanically. The simplest method to distinguish between a "virus"-induced tumor and a tumor transplantable only in the form of intact cells seems to be this test of cell-free homogenates for their ability to produce tumors.

We developed a method to isolate nuclei from the cells of the mouse ascites tumor. The cells were placed in distilled water, in which they swell; they were centrifuged and again placed in distilled water. If this process was repeated 3–4 times, some of the swollen cells began to lose cytoplasm. This removal of cytoplasm may be easily completed by the addition of a surface-active substance, such as digitonin. This method enables us to obtain isolated nuclei in a morphologically pure state (see Fig. 8). These nuclei produce no tumors after transplantation, in agreement with the behavior of cell nuclei prepared by homogenization. Although pure nuclear fractions may be obtained in this way, this method is not suitable for chemical investigations on nuclei, because a great loss of substances must occur during the preparation.

During the repeated process of swelling, the tumor cells lose granules from their cytoplasm—large as well as small ones. Thus, we can obtain cells with a nucleus and cytoplasm, but with a gradual loss of structural cytoplasmic components (Fig. 7). If the swelling and centrifuging of the cells took place only 2 or 3 times, the cells were still able to produce an ascites tumor identical to that from untreated cells. However, cells treated 4 times (with their cytoplasm almost free of particulates) gave no tumors after injection into mice. Thus, we can conclude that reconstruction of a complete cell is impossible from a cell with nucleus and cytoplasm, but depleted of mitochondria and microsomes. Although we must realize that the nucleus of these cells may be damaged by the repeated process of swelling, we can consider these findings as a hint that mitochondria can arise only from mitochondria.

In the preparations of cells swollen 2 or 3 times, the phase contrast microscope shows granules still inside the cells as well as between the cells. The positive tumor-takes of these preparations suggested experiments on the behavior of mixtures of particulate-free cells and fractions with particulates. Isolated particulate fractions of this tumor produced no tumors and neither did particulate-free cells, as mentioned above; but when we injected into the same mouse particulate-free cells and particulate fractions or a mixture of them, we observed the production of tumors. We may evaluate such a result as positive only if the cell fractions used in the experiment are unable to produce tumors when injected alone. Thus, in every experiment controls with the individual fractions are necessary. Out of seventeen experiments, six gave a positive result: the components alone gave no tumor, the mixture produced tumors. In five experiments, one component alone produced tumors; therefore, these experiments cannot be evaluated. In six experiments we obtained tumors neither from the components alone nor from the mixture. Regarding the fragility of the fractions

and the many possibilities of injuring them in their purification, negative results are of a high probability. If we consider the positive results to be reliable, there exists the possibility of recombination of plasma granules with particulate-free cells. Mixtures of particulate-free cells and complete homogenates also resulted in positive takes. The supernatant fluid of homogenates of tumor cells (free from structured particles) was unable to produce tumors when combined with particulate-free cells. Thus, we think that the particles of the cytoplasm are important for the effect and not other substances such as proteins or compounds of lower molecular weight. At a temperature of +4 to 10° C., the cells of the mouse ascites tumor are stable for 6–8 days. At this temperature tumor cells dialyzed against distilled water showed no loss of transplantability. This demonstrates that substances of low molecular weight able to dialyze under these conditions can be reacquired from the body fluids.

The possibility of recombining tumor cell fractions can be regarded as an interesting fact in cancer research, if these data are reliable. Therefore, these experiments are now being repeated; we are trying to find reproducible conditions for the preparation of the cell fractions. The conclusions from these results may suggest new aspects concerning the mechanism of the formation of metastases and recurrences: perhaps such fragments of tumor cells can combine with scattered cells which have lost their capacity for growth and thus initiate neoplastic growth. An analogy is given to the induction of tumors by viruses—but no more than an analogy. The development of grafted tumors can be accelerated by injections of homogenates of the same tumor type. In the mouse ascites tumor, a wave of mitoses occurs several hours after the injection of a homogenate of this tumor. Among the fractions of the homogenate, the mitochondrial fraction is the most effective, while the nuclei and the supernate are less effective.

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Fig. 1.—Untreated culture of mesenchymal fibroblasts. Living cells. ×240.

Fig. 2.—Chicken heart fibroblasts 24 hours after the addition of 0.1 μg/ml N-propylcolchicamide. Living cells. ×240.

Fig. 3.—Untreated fibroblasts in metaphase. Ehrlich-Biondi stain. Phase contrast microscope. ×540.

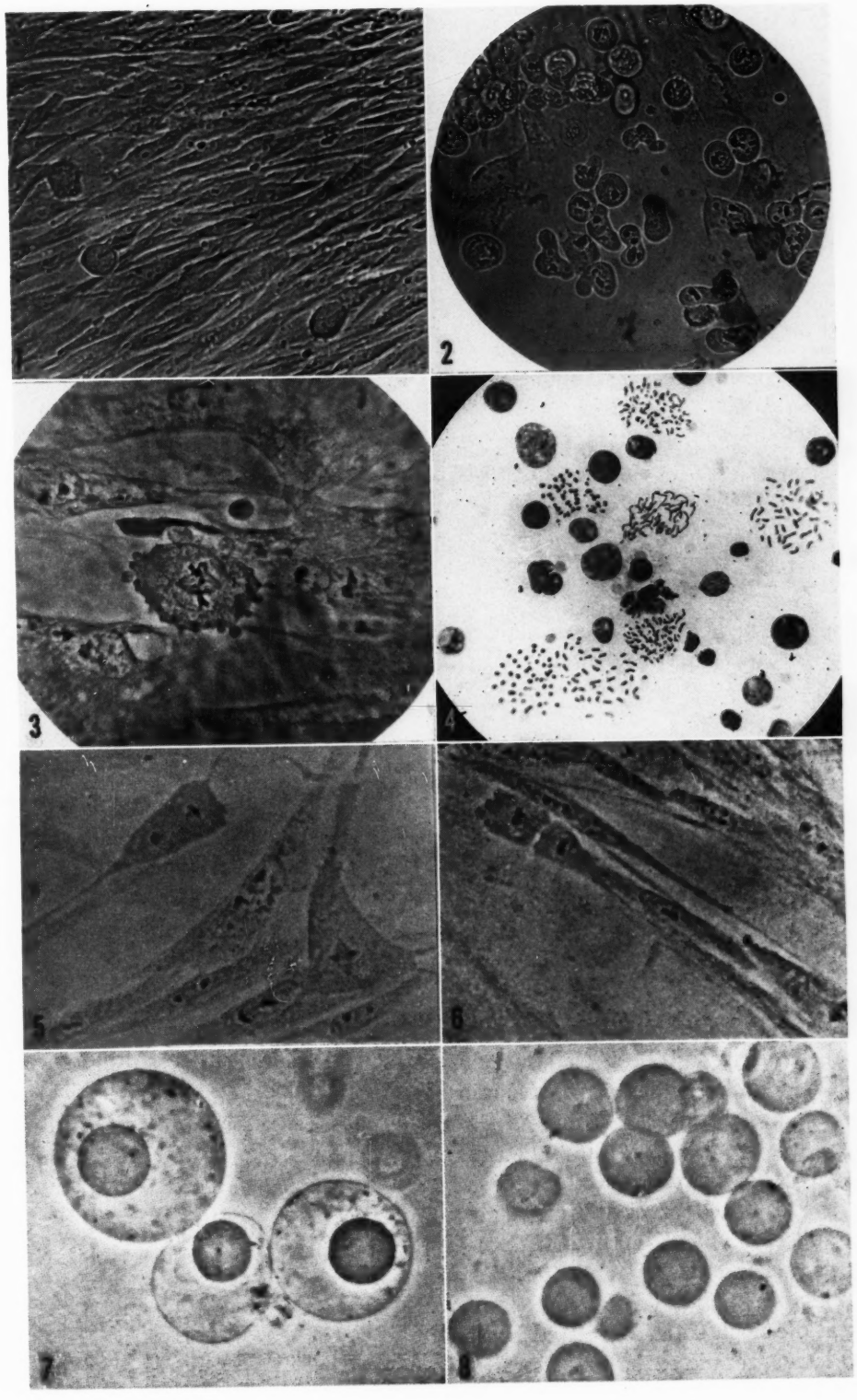
Fig. 4.—Cells of the mouse ascites tumor 24 hours after the injection of 4 μg. N-methylcolchicamide. Arrested metaphases with scattered chromosomes. Feulgen stain. ×650.

Fig. 5.—Fibroblasts 3 hours after the addition of 1 mg/ml ATP (sodium salt). Ehrlich-Biondi stain. Phase contrast microscope. ×480. Metaphase in a triangular cell, telophase in an elongated cell.

Fig. 6.—Same conditions as Figure 5. Type of a cell division by "tearing." A metaphase in a spindle-shaped cell.

Fig. 7.—Cells of the mouse ascites tumor deprived of granules (prepared by swelling, repeated 4 times). Compare the cell size with Figure 4. Phase contrast microscope. ×630.

Fig. 8.—Isolated nuclei from swollen cells. ×630.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Induction of Accessory Limbs and of Sarcoma in the Newt (*Triturus viridescens*) with Carcinogenic Substances*†

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Among adult vertebrates, only some of the amphibia are capable of regenerating their limbs. Several years ago experiments were begun in an attempt to induce neoplasia in limb tissues capable of such regeneration. The common newt, *Triturus viridescens*, was used because of its hardiness and availability. The injection of a variety of supposedly carcinogenic substances into the forelimb of over 500 newts resulted in the development of malignant neoplasms in only two animals. Both neoplasms were sarcomas, and both followed the injection of methylcholanthrene. However, a large number of animals developed non-neoplastic "new growths." These consisted of well organized reduplications of the injected limb. The results of the early experiments have been briefly summarized in preliminary reports (2, 3).

The experiments to be reported here deal with the specificity of the stimuli necessary for the induction of the accessory limbs and of the sarcomas, and with the nature of the accessory limbs.

MATERIALS AND METHODS

Adults of *Triturus viridescens*, caught in ponds near Philadelphia, were maintained in aquaria. Some were kept at 20°–22° C. in semi-darkness, in aerated water, and were fed on Tubificid worms. Others were kept in well lighted rooms at 25°–30° C. in standing water containing floating plants, and were fed raw muscle or liver. There was no striking difference in response to injections among the two groups; in general, the latter group was better nourished and developed accessory growths earlier.

The substances tested included carcinogenic Pennsylvania coke oven tar, methylcholanthrene, benzpyrene, acetylaminofluorene, scarlet red, vaseline, beryllium hydroxide, and particles of two amphibian neoplasms. These substances were injected directly into the loose subcutaneous and muscular tissues of the proximal region of the forelimb in amounts of 0.005–0.02 cc., with a tuberculin syringe and an 18- to 23-gauge needle. The animals were anesthetized by placing them in ether vapor.

Coal tar, when injected alone, was first heated to 100° C. in order to dissolve its precipitated constituents. It was then drawn into a warm syringe and chilled under the water tap for injection. When pure carcinogens were added to tar, they were dissolved in heated tar at about 120° C., and the mixture chilled in a syringe, as stated above. Details of the preparation of fractions of coal tar for injection are described later. Carcinogens in olive oil or vaseline were dissolved and injected in the same way. Rapid chilling resulted in fine, easily injectable crystals of those carcinogens that tended to crystallize out at room temperature. In one experiment, methylcholanthrene crystals were ground in a mortar with sufficient olive oil to make a paste, which was then injected into the forelimb with an 18-gauge trocar.

Beryllium hydroxide was prepared by adding sodium hydroxide to beryllium nitrate. The flocculent precipitate was purified by washing and by dialysis against distilled water. A 5 per cent suspension was injected in amounts of 0.02 cc. [1 mg. $Be(OH)_2$] into the forelimb.

Frog kidney carcinoma and sarcoma of the newt were injected as tumor particles, using 18-gauge trocars.

Deep thermal injury to the forelimb was caused by contact with a steel wire heated to about 500° C.

Fracture of the humerus was produced by means of a small rongeur, through a longitudinal incision.

EXPERIMENTAL RESULTS

I. Induction of Accessory Limbs and of Sarcoma

Experiments with coal tar and pure carcinogens.—Table 1 shows the incidence of accessory limbs and of sarcoma among newts receiving a single injection into the proximal region of the forelimb of various substances or subjected to severe thermal or mechanical injury of the same region. The variety of accessory limbs or limb parts induced is illustrated by representative photographs in Figures 1–4.

In the first attempts at tumor induction in newts with tar, 4 per cent each of methylcholanthrene, benzpyrene, acetylaminofluorene, and scarlet red were added to it. As seen from Table 1, 40 per cent of animals injected with this mixture developed accessory limbs. In order to determine the active ingredient, injections were made with components of the mixture. Tar alone proved less

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active than the mixture, inducing accessory limbs in 26 per cent of animals. Tar containing 4 per cent added methylcholanthrene or benzpyrene or acetylaminofluorene was even less active than tar alone, while tar containing 4 per cent added scarlet red in olive oil was as active as the original mixture. However, 4 per cent scarlet red in olive oil was inactive in ten animals.

TABLE 1

INCIDENCE OF ACCESSORY LIMBS AMONG NEWTS INJECTED INTO THE PROXIMAL REGION OF THE FORELIMB WITH VARIOUS CARCINOGENIC OR NONCARCINOGENIC SUBSTANCES, OR SUBJECTED TO LOCAL INJURY
(Animals that developed sarcoma are designated by *S*)

Material injected*	No. animals injected†	Animals developing accessory limbs		Time of appearance of first accessory limb (days)
		No.	Per cent	
Coal tar	76	20	26	41
Coal tar+4 per cent MCA, BP, AAF, and SR	50	20	40	42
Coal tar+4 per cent MCA	20	4	20	49
Coal tar+4 per cent BP	19	2	11	87
Coal tar+4 per cent AAF	20	3	15	49
Coal tar+4 per cent SR	20	8	40	49
Olive oil+4 per cent MCA	4	1	25	300
Olive oil+4 per cent AAF	10	0	0	
Olive oil+4 per cent SR	10	0	0	
Vaseline+4 per cent BP	23	0	0	
Vaseline	22	1	5	300
MCA crystals wet with olive oil	34	1 *SS*‡	3	130
Beryllium hydroxide	18	6	33	130
Rana pipiens kidney carcinoma	75	3	4	200
Triturus viridescens sarcoma	274	0	0	
Deep burn of forelimb (not injected)	21	0	0	
Fracture of humerus (not injected)	23	0	0	

* MCA=20-methylcholanthrene; BP=3,4-benzpyrene; AAF=2-acetylaminofluorene; SR=scarlet red.

† Animals surviving 50 or more days.

‡ Sarcoma developed in two animals. One of the tumors arose at the base of the accessory limb.

Acetylaminofluorene in olive oil and benzpyrene in vaseline were also inactive, while vaseline alone induced a single accessory digit-like growth after 300 days. Methylcholanthrene in olive oil induced a small accessory limb with two digits in one of four animals after 300 days. Of 34 animals, each receiving approximately 1 mg. of methylcholanthrene crystals wet with olive oil and injected with a trocar, one developed an accessory limb without digits (Fig. 8). However, a sarcoma developed in this limb a year later (Fig. 9). Another animal developed sarcoma 168 days after injection, but an accessory limb did not form. These two animals are described in Part III of "Experimental Results."

Beryllium hydroxide (Table 1) induced accessory limbs in six of eighteen animals injected.

Experiments with tumor tissue.—Particles of spontaneous carcinoma of the kidney of the leopard frog, a tumor probably caused by a virus (14), induced three accessory limbs among 75 animals injected (Table 1). In contrast, none of 274 animals injected with particles of the two strains of transplantable methylcholanthrene-induced sarcoma referred to above developed accessory limbs.

Effect of thermal and mechanical injury.—No accessory growths developed on the forelimbs of 21 animals burned with a hot wire, so as to involve the humerus, and of 23 animals in which the humerus was fractured with a rongeur and allowed to heal.

The incidences listed in Table 1 refer to all types of accessory limb structure that could be identified as such. The simplest growths were single digit-like projections arising from the site of injection (Fig. 4). Others ranged in complexity up to doubly reduplicated limbs, i.e., two accessory forelimbs growing from the injection site. However, it is noteworthy that only those animals receiving coal tar developed the growths early (41–87 days); and only those receiving coal tar, alone or with added carcinogens, or beryllium developed the growths in high incidence. Furthermore, only tar and beryllium produced complex growths. The other accessory growths were relatively simple, having no more than two digits, and appeared late (130–300 days).

Experiments with various fractions of coal tar.—Berenblum and Schoental (1) have shown that petroleum ether effectively extracts benzpyrene and at least one other potent carcinogen from tar. Such extracts are clear yellow solutions that, on evaporation, leave a yellow-brown semicrystalline residue.

Pennsylvania coke oven tar (200 ml.) was extracted with petroleum ether (500 ml.) by shaking the two together at intervals over a period of 4 days at 37° C. The extract was evaporated to dryness. Ten grams of the residue were dissolved in 100 ml. of petroleum ether, and the solution was passed through an adsorption column containing 250 gm. of light powdered magnesia. Petroleum ether was continuously added to the top of the column. A first fraction (1-A) of 250 ml. and a second fraction (1-B) of 500 ml. were thus collected. A third fraction (1-C) of 500 ml. was obtained by adding benzene to the column. Each fraction was evaporated on a water bath, the residue dissolved in 2 parts of vaseline at 120° C. and chilled in a syringe under the water tap to

form a jelly-like mass for injection. Each fraction thus prepared was injected in amounts of 0.01 cc. into the proximal region of the forelimb of sixteen animals.

The results are shown in Table 2. Fraction 1-A,

TABLE 2

INCIDENCE OF ACCESSORY LIMBS AMONG NEWTS INJECTED INTO THE PROXIMAL REGION OF THE FORELIMB WITH VARIOUS FRACTIONS OF COAL TAR

MATERIAL INJECTED	No. ANIMALS INJECTED*	ANIMALS DEVELOPING ACCESSORY LIMBS No.	Per cent	TIME OF APPEARANCE OF FIRST ACCESSORY LIMB (DAYS)
Adsorption fractions:				
1-A	16	0	0	
1-B	16	4	25	52
1-C	16	10	63	52
Industrial tar fractions:				
Tar before distillation	13	6	46	54
"Middle Creosote Oil" (287°–343° C.)	16	7	44	54
"Heavy Creosote Oil" crystals (343°–416° C.)	16	5	31	100
"Heavy Creosote Oil," mother liquor	7	5	71	54
"Fuel Pitch" (residue in still)	16	10	63	90

* Animals surviving 50 or more days.

the least adsorbable on magnesia, was inactive; Fraction 1-B induced accessory limbs in four animals; and Fraction 1-C was active in ten animals.

The association of activity with high adsorbability suggested that activity was due to compounds of high molecular weight. Consequently, several tar distillates available in industry were tested. A sample of Pennsylvania coke oven tar and some of the fractions derived from it by large-scale distillation were obtained.[1] These were (*a*) "Medium Creosote Oil," distilling between 287° and 343° C.; (*b*) "Heavy Creosote Oil," distilling between 343° and 416° C.; and (*c*) "Fuel Pitch," the residue left in the still. Petroleum ether extracts were prepared from the tar and its fractions and were evaporated. The "Heavy Creosote Oil" residue separated into yellow crystals and a brown mother liquor. Crystals and mother liquor were tested separately. Each fraction was dissolved in 3 parts of vaseline, cooled rapidly, and injected in amounts of 0.01 cc. into the proximal region of the forelimb.

The results are shown in Table 2. All the extracts were active, especially those of the higher boiling tar fractions and of fuel pitch. The mother liquor of "Heavy Creosote Oil" proved to be very toxic, and more than half of the injected animals died within 2 weeks.

The high activity of all the fractions, as shown in Table 2, makes it difficult to ascribe the accessory limb induction to one or a few compounds present in tar. It is possible that, during industrial distillation, changes such as cracking and recombination of molecules occur, so that large molecules appear in all fractions. At any rate, it is of interest that tar fraction 1-C and fuel pitch both induced the same high incidence of accessory limbs (63 per cent), a result consistent with the hypothesis that at least some heavy molecules in tar show a high degree of activity.

There was some indication that 25 per cent fuel pitch extract in vaseline, when stored in the refrigerator, loses its activity. A sample stored for 5 months was tested in the following concentrations of fuel pitch extract in vaseline: 25 per cent, 12.5 per cent, and 6.25 per cent, which gave, respectively, incidences of 1 in 20, 2 in 19, and 1 in 20 newts with accessory limbs. These incidences are of the order observed with vaseline alone (Table 1).

Effects of injection of sites other than the forelimb.—The tar mixture containing 4 per cent added methylcholanthrene, benzpyrene, and scarlet red was injected in amounts of 0.1 cc. into the hind limb of twelve newts. One animal lost the injected limb, due to infection and necrosis, and then regenerated a double hind limb, the two members of which were mirror images of each other. No growths of any kind appeared among the remaining eleven animals.

The same mixture was injected into the side of the tail of twelve newts. Six developed flat shelf-like projections that extended laterally from the injection site and had the gross and microscopic appearance of diminutive accessory tails.

Injection of the tar mixture into the back of twelve newts was followed by the development of raised pigmented nodules containing the injected material. No accessory structures or neoplasms developed.

II. PROPERTIES OF ACCESSORY LIMBS

Mode of development and structure of accessory limbs.—Examination of a number of microscopic sections at various periods after the injection of tar into the forelimb showed the following changes: there was at first an apparently nonspecific inflammatory reaction around the injected mass that lasted from a few weeks to several months. During this time there was a concomitant degeneration of striated muscle and resorption of bone. These changes were very similar to the "dedifferentia-

[1] Kindly supplied by the Barrett Division, Allied Chemical and Dye Corporation.

tion" that is said to occur after amputation of a limb and before a regeneration blastema has formed (7). At any rate, the differentiated tissues of the injected region were soon replaced by a tissue resembling regeneration blastema. It was this tissue that eventually gave rise to the muscle and bone of the accessory limb, with accompanying ingrowth of nerve from the host limb and migration and growth of host limb epithelium over the accessory growth. The first readily identifiable limb structure to develop was a cone of cartilage, such as is shown in Figure 6. It was surrounded by muscle cells in various stages of differentiation, together with a few nerve fibrils. Further growth and differentiation might or might not occur. When it did, organization was of such a degree that the resulting accessory limb tissues could not be distinguished from normal limb tissues (Fig. 5).

In some animals the initial degeneration (or "dedifferentiation") of muscle and bone were very extensive; sometimes fracture of the humerus occurred at the site of injection. However, destruction of tissue was not directly related to accessory limb formation. Injury to tissue was as severe in the methylcholanthrene series, where incidence of accessory limbs was low, as in the tar series, where it was high. The three tar fractions, 1-A, 1-B, and 1-C (Table 2), produced about the same degree of local tissue destruction but were quite different as regards their ability to induce accessory limbs. Furthermore, thermal injury or fracture of the humerus did not result in accessory limb formation (Table 1), nor did any accessory limbs develop among many animals whose limbs were partly destroyed by a chronic fungus-like infection.

It is noteworthy that the accessory limbs or limb parts in no case became larger than their normal counterparts. Also, the induced growths always consisted of reduplications of structures found *distal* to the point of injection, e.g., digits, carpal bones, and radius and ulna. In these respects, and in their high degree of tissue organization, the growths resembled regenerates such as might be expected following amputation of a limb at the point of injection.

Fate of accessory limbs after amputation and transplantation.—The foregoing observations suggested that the accessory "new growths" represented alterations of the normal regeneration mechanism and were not neoplasms. It was possible, however, that neoplastic or preneoplastic cells were present in them, cells somehow held in check by the actively differentiating blastema. For example, the methylcholanthrene-induced papillomas of rabbits, of apparently "benign" nature, extensively investigated by Friedewald and Rous (10) and others, provided a basis for this concept. These tumors may develop into invasive carcinomas when subjected to trauma or other nonspecific injuries. In the present experiments the effects of trauma and of a new tissue environment were tested by amputating the growths and observing their regeneration, as well as by transplanting fragments of the growth to different sites in the same animal or to different animals.

Amputation of host limbs.—It was of interest first to determine whether the presence of an accessory limb changed the regenerative qualities of the host limb. The forelimbs of six newts, each bearing an accessory limb, were amputated *proximal* to the accessory growth. All six animals regenerated normal limbs without accessory growths. Subcutaneous autotransplants of accessory limb tissue to the opposite limb and side failed to grow during 1 year of observation of all six animals.

Amputation of accessory limbs.—In the remaining experiments the accessory limb itself was amputated, 0.5–1 mm. from its base.

The regenerates appearing after amputation of accessory limbs were varied, but in general they either approximated the structure of the amputated growth or were less complex. These relations are shown in Table 3, where the number of digits on the accessory limb before its amputation is compared to the number on the regenerate arising from the amputation stump. In all animals the regenerates ceased growing at or before the time they reached the size of corresponding normal structures. No changes suggestive of neoplasia were seen in any of them.

TABLE 3

TYPES OF REGENERATES RESULTING FROM AMPUTATION OF ACCESSORY LIMBS

Comparison of Number of Digits on Amputated Limb to Number on Regenerate

No. animals	No. digits on accessory limb	No. digits on regenerate*
1	7	3
1	6	7
5	4	4, 2, 2, 2, 1
1	3	3
5	2	3, 2, 2, 1, 1
2	1	1, no regenerate

*I.e., on accessory limb regenerating after amputation.

In fifteen animals, fragments of the accessory limb were autotransplanted to the opposite limb and the body wall, while the growths removed from two animals were transplanted to a total of ten other newts. Only one of the transplants be-

came established. This was an autotransplant to the opposite limb. A small conical projection slowly grew from the injection site and reached a height of 1 mm. after 3 months. It grew no more during 1 year of observation. None of the other transplants showed evidence of becoming established. These results contrast with those obtained with the two methylcholanthrene-induced sarcomas, both of which could readily be transplanted by the same technic.

III. Induction of Sarcoma by Methylcholanthrene

Two sarcoma strains arising from tumors in newts injected with methylcholanthrene (cf. Table 1) are being maintained by transplantation in other newts. Their properties will be described in detail in a later publication. The induced tumors are shown in Figures 9 and 10, a transplanted tumor in Figure 11, and the microscopic appearance of the tumors in Figures 12, 13, and 14.

It is of interest that both of the sarcomas occurred in male newts, whereas the incidence of accessory limbs was the same in males and females. Curiously, the sarcomas when transplanted have taken better in females.

As many injected animals as possible are being kept for extended periods in order to determine whether other neoplasms arise, particularly in animals bearing accessory limbs. Over 230 have been observed for 1–2 years.

DISCUSSION

When newts that have been taken from their natural habitat are examined with care, an occasional animal bearing an accessory or reduplicated limb is found. In this laboratory, only two among some 1,500 *T. viridescens* have been seen, one an imperfect accessory growth on the forelimb and the other a reduplicated hind limb.

Experimentally, accessory limbs have been produced by severe injuries that apparently involved nerves. Detailed accounts of the induction of such growths in salamanders by the surgical deviation of limb nerves are given by Guyénot and Schotté (12) and by Guyénot *et al.* (11) and others. If a nerve is shifted from its normal course and made to emerge near the skin, an accessory limb frequently develops near its tip. Fibers growing from the end of the nerve seem to be capable of initiating the formation of blastema.

It is possible that nerve deviation has played a part in the present experiments as a consequence of the destruction and regeneration of nerve. An injured, inflamed, or scarred region may prevent regenerating nerve from following its original course. The new twigs may then be forced far enough out of their original path to emerge near the skin, under conditions similar to those attending surgical deviation. If nerve deviation is involved in the present experiments, it appears necessary to assume that the limb-inducing substances affected in some chemical manner the directional growth of nerves, since other substances incapable of limb induction were just as injurious to tissue, and presumably to nerves, as those known to be active.

Nerve is necessary but not of itself sufficient for the formation of limb regenerates (8, 16, 18). Cells capable of forming a blastema are also required, and these are derived from the limb itself. This fact has been demonstrated by Butler (6) in experiments involving the inactivation of the blastema-forming cells by means of roentgen rays. Similar inactivation was achieved by Thornton, using beryllium (17). However, Brunst (4) and Brunst and Figge (5) have shown that roentgen rays in small doses are capable of *stimulating* the blastema-forming tissue, and have in this manner induced secondary (accessory) limbs and tails in young axolotls. In their experiments it is improbable that nerve deviation occurred. The results lend support to the hypothesis that in the present experiments a direct stimulation of blastema-forming tissue by the injected substances occurred.

There is little evidence to connect the accessory growths and sarcoma in a causal manner. Only methylcholanthrene proved carcinogenic, yet it induced relatively few accessory limbs. Coal tar and certain fractions of tar, and beryllium, which is known to induce osteogenic sarcoma in rabbits (9), induced a high incidence of accessory limbs but no neoplasms. It seems likely that, of substances carcinogenic in mammals, some may induce only blastema formation in the newt or may be inactive, whereas methylcholanthrene can induce neoplasia as well. It is of interest that beryllium is a powerful inhibitor of alkaline phosphatase (13), an enzyme known to be of importance for the growth of bone, which forms a prominent part of the accessory limbs.

A number of investigators have attempted to induce neoplasia in cold-blooded vertebrates by means of chemical agents. In a review published in 1949 Lucké and Schlumberger (15) state: "No convincing evidence has been produced that true neoplasia has been induced by any of the chemicals used in any cold-blooded vertebrate."

In the present experiments such induction has been accomplished.

SUMMARY AND CONCLUSIONS

Adults of the common newt, *Triturus viridescens*, were given injections of various supposedly carcinogenic and noncarcinogenic substances into the proximal region of the forelimb. Other newts were subjected to thermal and mechanical injury of the same region.

Certain fractions of coal tar, or tar either alone or with added methylcholanthrene, benzpyrene, acetylaminofluorene, or scarlet red, induced the formation of accessory limbs in a high incidence (11–63 per cent). The accessory limbs grew from the site of injection and consisted of reduplications of normal limb structure found distal to the point of injection.

Of other substances tested that did not contain tar or tar fractions, only beryllium hydroxide induced a high incidence of the accessory limbs (33 per cent).

A few accessory limbs were induced by vaseline and by particles of carcinoma of the kidney of the leopard frog.

Benzpyrene, acetylaminofluorene, scarlet red, Triturus sarcoma, and local injury failed to induce accessory limbs.

Sarcoma developed in two animals that had been injected with methylcholanthrene; in one the sarcoma was at the base of an accessory limb. Both sarcomas were highly invasive and have been serially transplanted in other newts.

In contrast, the accessory limbs consisted of well organized, apparently normal limb tissues, and could not be transplanted.

It is concluded that some carcinogenic substances are capable of inducing not only neoplasia but highly organized growth as well.

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Fig. 1.—Adult *T. viridescens* injected into proximal region of right forelimb 149 days previously with coal tar containing added methylcholanthrene, acetylaminofluorene, and scarlet red. The accessory limb appeared as a bud in the right elbow after 50 days and developed rapidly. The limb was capable of independent motion, and the animal used it in walking. ×4.

Fig. 2.—Adult *T. viridescens* injected 50 days previously with coal tar. The growth is beginning to develop four digits. The forelimb was amputated 2 mm. proximal to the accessory growth 5 days after the photograph was taken. Subsequently to this the entire forelimb regenerated normally (50 days), without development of any accessory growths. ×4.

Fig. 3.—Adult *T. viridescens* injected 385 days previously with coal tar containing 4 per cent added benzpyrene. Two or three digits appeared at the site of injection after 90 days; in total, six were present after 150 days, and eight were present after 350 days. ×4.

Fig. 4.—Adult *T. viridescens* injected 69 days previously with the same tar mixture as the animal shown in Figure 1. The accessory growth is a single conical projection. After 150 days it increased several times in size, developed two digit-like projections at its tip, and ceased growing .×4.

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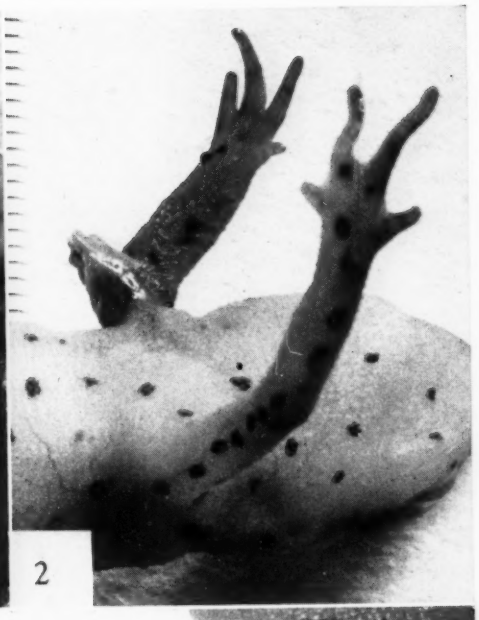

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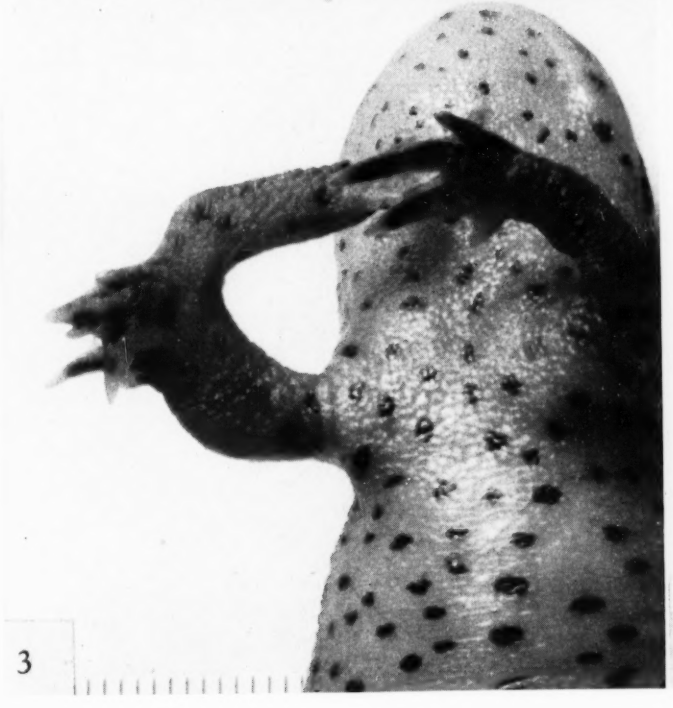

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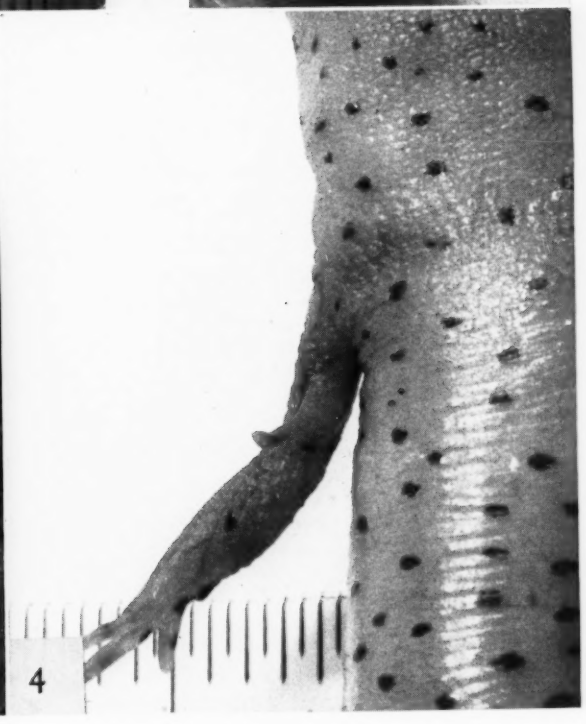

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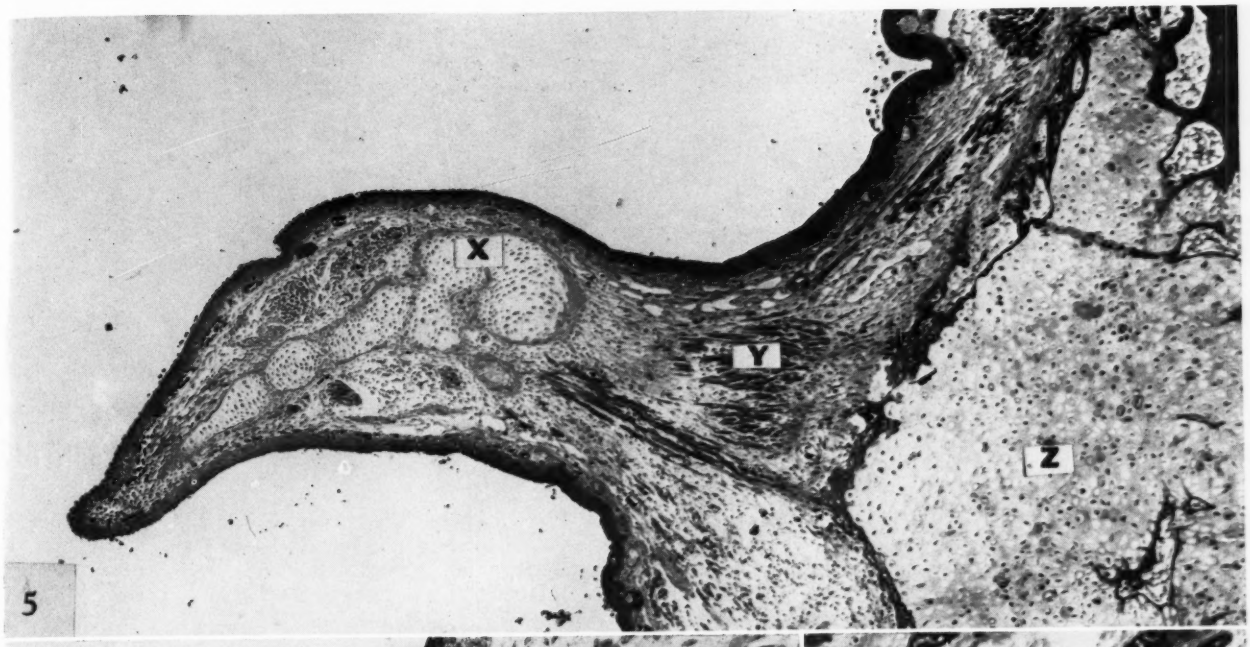
FIG. 5.—Section through a well-developed accessory limb, having 4 digits, induced by injection of coal tar 92 days previously. The carpal cartilages are shown at *X*. A longitudinal section of one digit extends distally from them. Striated muscle and nerve fibers are present at *Y*. The humerus of the host limb, from which the accessory limb arises, is shown at *Z*. The normally occurring bone of the humerus has largely been replaced by newly formed cartilage. Holmes' silver impregnation. ×28.

FIG. 6.—Section through a cone-shaped poorly differentiated accessory limb induced by coal tar injected 92 days previously. Its gross appearance was similar to the growth shown in Fig. 4. A central cone of cartilage is to be seen at *X*. Striated muscle is present at *Y*, and nerve fibers at *Z*. The growth is covered by mature skin. Holmes's silver impregnation. ×125.

FIG. 7.—Higher magnification of the region of striated muscle seen in Fig. 6 (*Y*). The striations are not very prominent, as the muscle is still in the process of differentiation. Holmes's silver impregnation. ×450.



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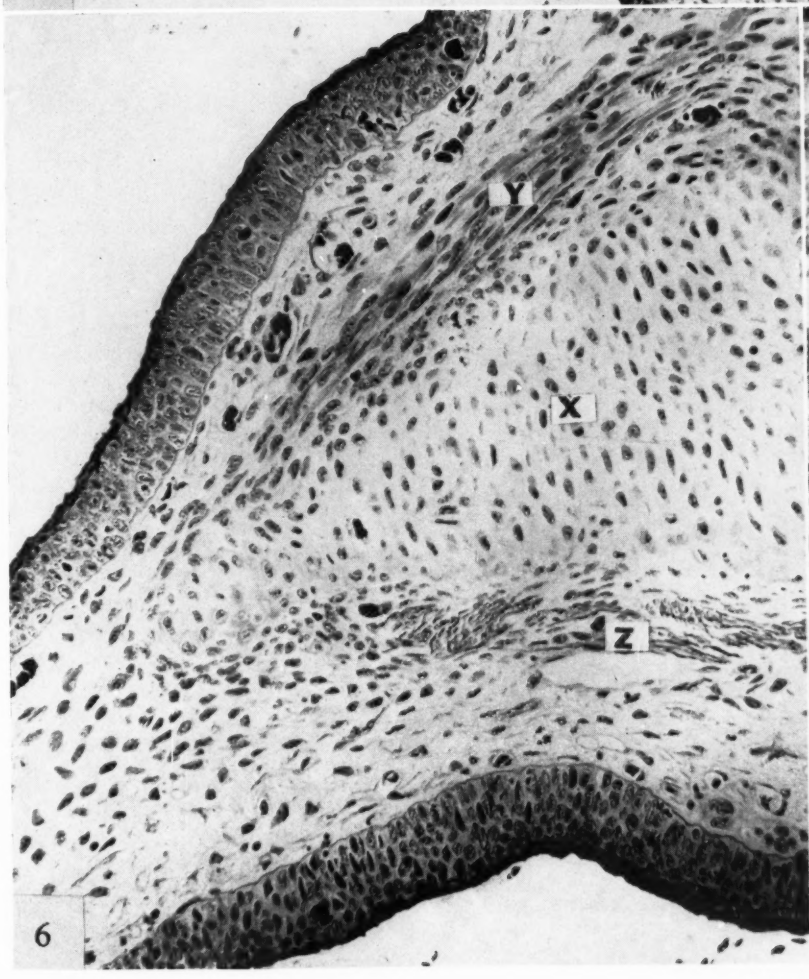

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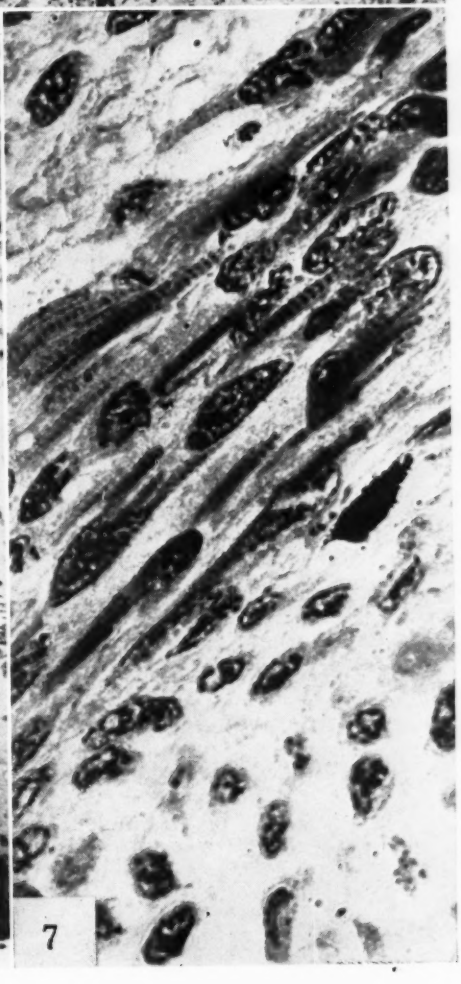

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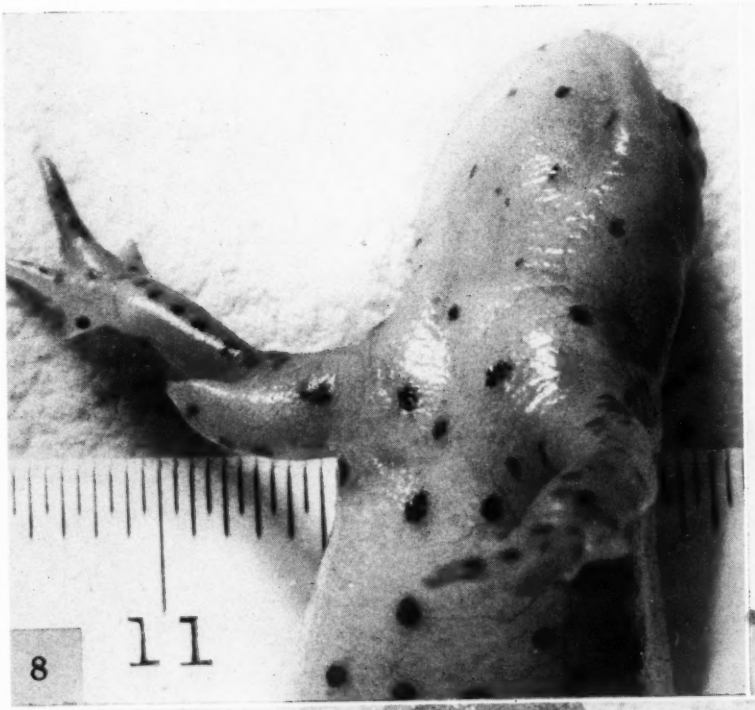
FIG. 8.—Adult *T. viridescens*. Cone-shaped growth from right forelimb and axillary region 168 days after injection of this site with approximately 1 mg. methylcholanthrene crystals wet with olive oil. This structure is an accessory limb without digits. ×4.

FIG. 9.—Same animal as shown in Fig. 8, but 488 days after injection. The base of the cone has become greatly thickened, and several engorged veins have appeared. There is also swelling of the right forelimb and of the right occipital region. The animal was sacrificed 2 days later. Upon microscopic examination the swelling was found to be an invasive sarcoma (Sarcoma 5) that had arisen at the base of the accessory limb and had extended to the base of the skull. ×4.

FIG. 10.—Adult *T. viridescens* injected 168 days previously into the proximal region of the right forelimb with approximately 1 mg. methylcholanthrene crystals wet with olive oil. The entire right forelimb shows nodular thickening, the swelling increasing proximally, so that limb and body join in a fusiform manner. The animal was sacrificed 3 weeks later and the tissues examined microscopically. The entire swollen region was found to be invaded by sarcoma (Sarcoma 27). ×4.

FIG. 11.—First passage of sarcoma shown in Fig. 9, 133 days after injection of tumor fragments into the right forelimb. ×4.



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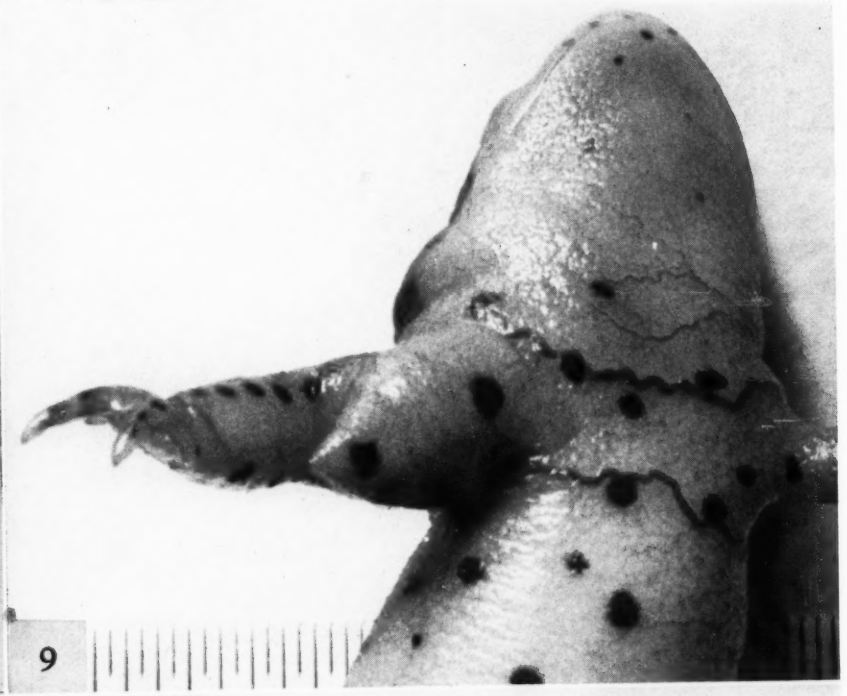

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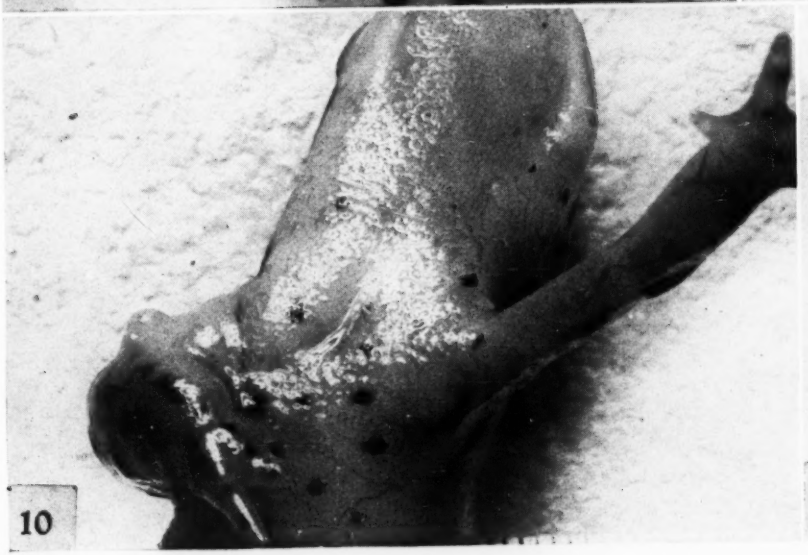

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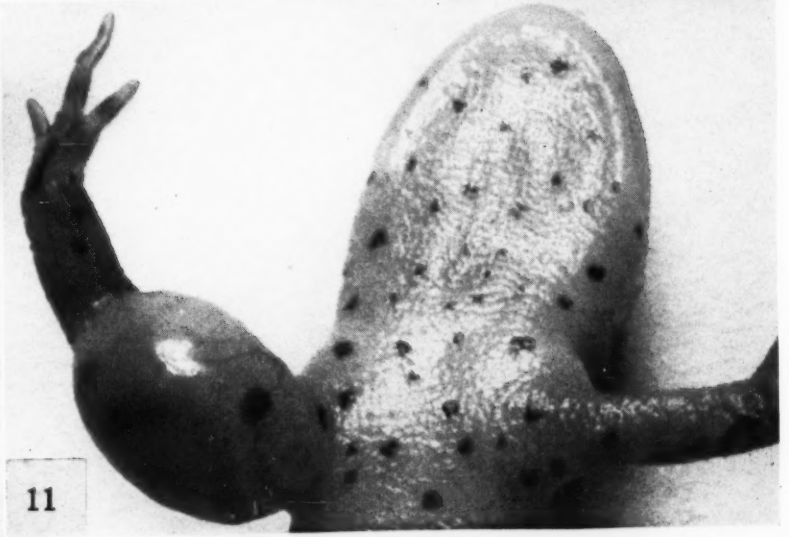

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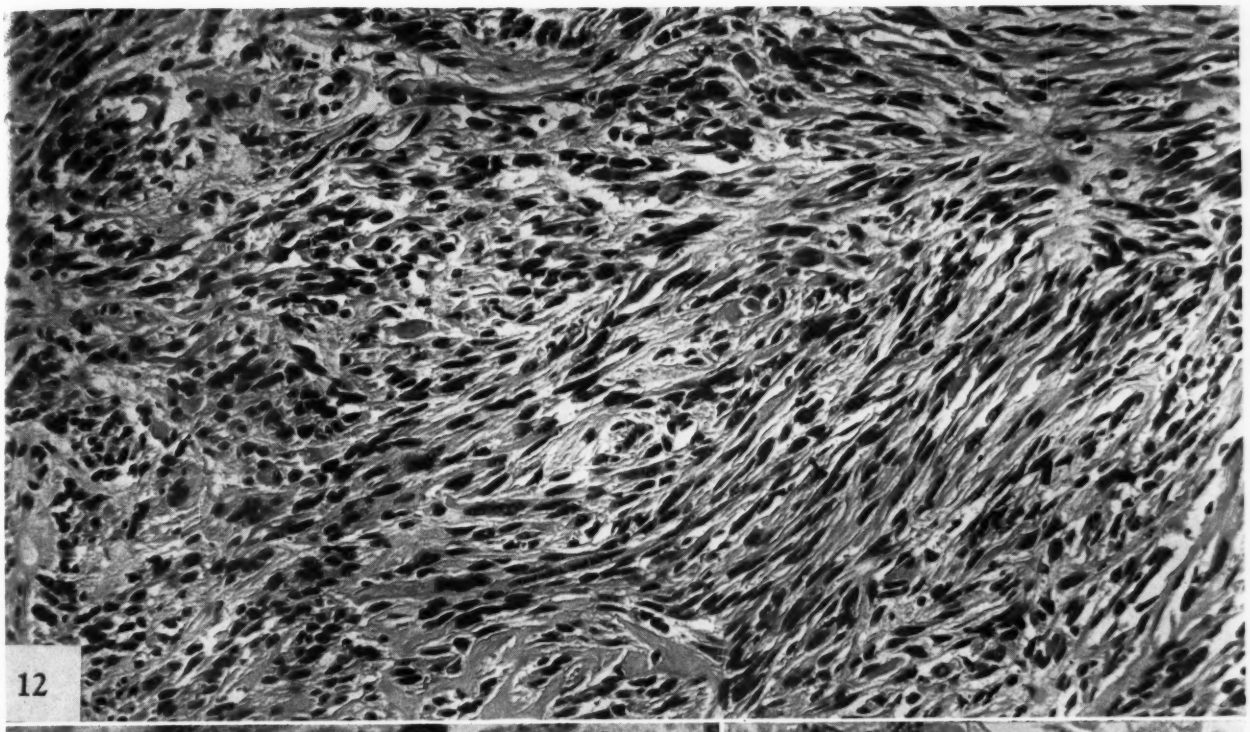
FIG. 12.—Section of Sarcoma 27 (see Fig. 10), showing the predominance of spindle-shaped cells and their arrangement in whorls. Hematoxylin and eosin. ×100.

FIG. 13.—First passage of Sarcoma 27. The tumor cells are shown invading muscle. One cell in the lower right-hand corner is in mitosis. Giemsa. ×450.

FIG. 14.—Third passage of Sarcoma 5. The central portion of a large tumor is shown. One cell just below the center is in mitosis. Giemsa. ×450.



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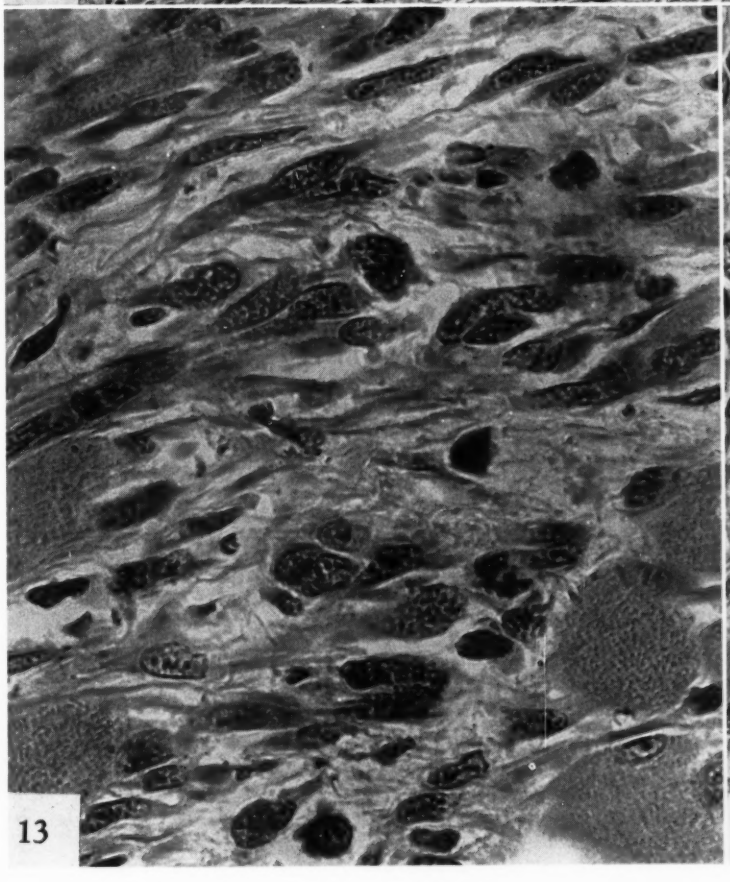

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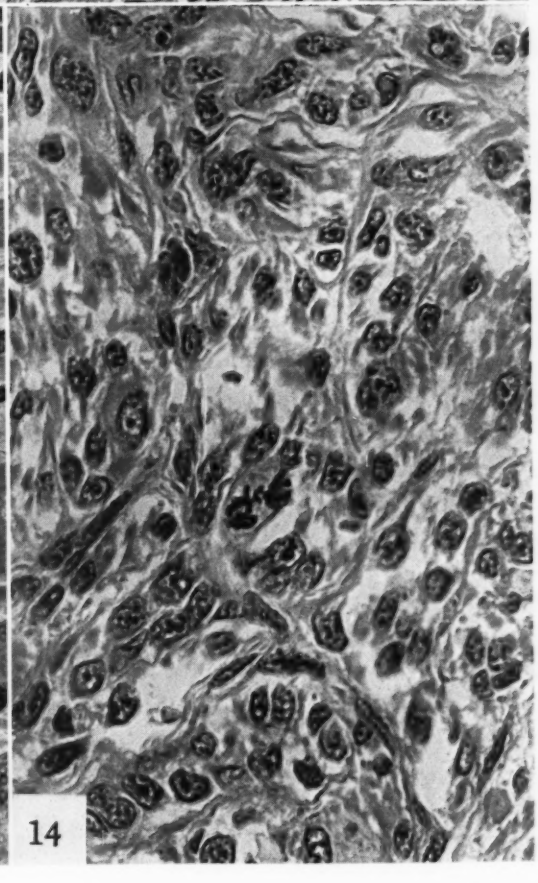

14

The Distribution of Administered Radioactive Rubidium (Rb^{86}) in Normal and Neoplastic Tissues of Mice and Humans*

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Previous observations (2) have shown a relatively small uptake of Rb^{86} by normal brain tissue after parenteral administration of Rb^{86} carbonate in the guinea pig and dog. This potentially low background would be useful in localizing intracranial tumors with sufficient isotope avidity. This problem was investigated by determining the uptake and distribution of parenterally administered Rb^{86} in normal and neoplastic tissues of mice and humans, with emphasis on neurogenic tumors. The results suggest the possible usefulness of this isotope for the localization of brain tumors.

MATERIALS AND METHODS

Rubidium is an alkali metal in the same series of the periodic table as lithium, sodium, cesium, and potassium, and has chemical and physical properties particularly similar to potassium. Naturally occurring rubidium is a mixture of stable Rb^{85} (72.8 per cent) and weakly radioactive Rb^{87} (27.2 per cent). Many artificially radioactive isotopes of this element have been produced; Rb^{86}, the isotope used in this study, has a half-life of 19.5 days and emits both beta (1.82 Mev) and gamma (1.08 Mev) rays.

Purified rubidium carbonate[1] was irradiated in the nuclear reactor at Oak Ridge, Tenn. The specific activity was initially 10 mc/0.65 gm. Trace quantities of cesium (Cs^{134}) were present.

All specimens except urine and blood were prepared for analysis by digestion in 2 N NaOH. An aliquot of each specimen was plated and dried in small metal planchets, and radioactivity was determined by means of a well shielded, mica-end window, TGC-1A Tracerlab G-M tube. Counting rates were sufficiently high so that the statistical error was less than 5 per cent. Appropriate corrections were made for geometry, internal absorption, and decay.

Toxicity.—The dose of $Rb_2^{86}CO_3$ for mice was calculated to contain sufficient radioactivity for accuracy of measurement, while avoiding toxic pharmacologic and radiation levels. In mice weighing 20 gm. a dose of 0.1 cc. of a 13 per cent solution (13 mg.) caused very prompt spastic paralysis, followed by death in more than 50 per cent of the animals. Within 5 minutes after intraperitoneal injection, spasm was apparent in the hind extremities and rapidly became generalized. Respiratory movements were slowed, labored, and gasping. The heart continued to beat for a few minutes after complete respiratory paralysis developed.

With a dose of 0.08 cc. of the 13 per cent solution (10.4 mg.), only a few animals displayed muscular distress, and these invariably recovered shortly. This dose was therefore not exceeded in the experiments with mice.

RESULTS

Distribution in Normal and Tumor-bearing Mice

A group of six Swiss white mice bearing Sarcoma 37, transplanted 3 weeks earlier, and a like number of control animals were given intraperitoneal injections of a solution of Rb^{86} providing approximately 8.2×10^6 counts per minute (approximately 8.5 μ). Rapid absorption from the peritoneal cavity had been demonstrated in the preliminary toxicity studies.

The animals were sacrificed 24 hours after administration of the isotope. Weighed portions of various tissues were prepared as described above. The results are presented in Table 1 and represent the average findings in each group.

The Sarcoma 37 in this host concentrated less rubidium than the brain. The presence of the

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[1] A. B. McKay Co., New York.

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neoplasm did not appreciably alter the distribution of the isotope in the other tissues as compared to that in the control animals. The highest uptake was demonstrated in the pancreas. The further order of decreasing avidity was: spleen, liver, muscle, heart, brain, and tumor.

Effect of Variation in Interval Following Transplantation of Sarcoma 37 upon Uptake of Rb^{86}

Mice bearing the transplanted Sarcoma 37 for varying periods of time were used to determine the influence, if any, of the "age" of the tumor upon the distribution of Rb^{86}. Administration of the isotope and analysis of tissues were carried out as in the previous experiment.

A greater uptake of Rb^{86} by the sarcoma was demonstrated during the first 16 days following transplantation (Table 2). The tumor:brain concentration ratio was 2.5:1 during this period, which corresponds to and may be related to the phase of maximum rate of growth of the tumor in this host.

TABLE 2

Distribution of Rb^{86} in Mice Bearing Sarcoma 37 for Varying Durations

(Per cent of dose/gm)

"Age" of tumor (days)	Pancreas	Spleen	Liver	Muscle	Heart	Brain	Tumor
7	2.5	2.7	2.5	2.1	1.9	0.62	1.5
16	2.9	2.5	2.3	1.6	1.2	0.57	1.5
27	2.7	2.1	2.7	1.9	1.9	1.0	0.72
38	3.0	2.6	2.5	2.3	2.2	1.1	0.78

Distribution of Rb^{86} and Cs^{134} in Mice Bearing Tumors of Nervous System Origin

Prior to determination of the distribution of Rb^{86} in a suitable patient with brain tumor, studies were conducted in animals bearing neurogenic neoplasms. Eight ABC strain mice with subcutaneously implanted neuroblastomas and eight C3H strain mice bearing astrocytomas, transplanted 3 weeks and 6 weeks earlier, respectively, were used.[2] Cs^{134} in the form of cesium chloride was also used in the experiment, because it was present in trace amounts in the irradiated Rb^{86}. One-half of the animals received each isotope. Fifty per cent of each group was sacrificed after 4 hours, the remainder in 24 hours.

In each group of animals (Table 3) the absolute uptake of both isotopes by tumor and brain tissues was greater at 24 hours than at 4 hours. In every instance, however, the ratio of Rb^{86} concentration in tumor:brain was greater at the 4-hour interval. In the astrocytoma-bearing animals the tumor to brain ratios of 10.1 and 11.3 for Rb^{86} and Cs^{134}, respectively, are significantly higher than were the ratios observed in the experiments with Sarcoma 37 and neuroblastoma.

TABLE 1

Distribution of Rb^{86} in Sarcoma-bearing and Control Mice

(Per cent of dose/gm)

Group	Pancreas	Spleen	Liver	Muscle	Heart	Brain	Sarcoma
Sarcoma 37 mice	2.6	2.3	2.3	1.7	1.3	0.60	0.48
Control mice	2.6	2.6	2.3	1.9	1.6	0.68	

As in earlier experiments, the pancreas had the highest uptake of rubidium. This was also the case with cesium. The latter isotope, however, was distributed in distinctly smaller amounts in the liver and muscle.

Studies in the Human

Case 1.—On December 1, 1951, a male patient (A. H.) underwent craniotomy for an intracranial neoplasm.[3] The clinical manifestations indicated an expanding lesion in the posterior fossa encroaching upon the cerebellum and brain stem. The preoperative prognosis was poor.

Approximately 50 minutes before the operation, an intravenous injection of a 13 per cent solution of $Rb_2^{86}CO^3$, containing 100 μc. of Rb^{86}, was administered. The withdrawal of specimens of blood at predetermined intervals was prevented by exigencies of the operative procedure and changes in the patient's condition. Urine was collected via an indwelling catheter.

Attempts were not made to localize the tumor by external counting devices or through use of a radio-sensitive probe. Biopsy specimens obtained during the operation and specimens of other tissues obtained at autopsy were analyzed for Rb^{86} content. Autopsy disclosed a primary carcinoma of the esophagus with metastases to the brain and to retroperitoneal lymph nodes. Death was due to encroachment of the tumor upon vital brain structures.

Urine.—The urinary excretion during the first $3\frac{3}{4}$ hours was 0.49 per cent of the administered dose of Rb^{86} in a volume of 255 cc.

[2] We wish to thank Dr. Sheldon C. Sommers, Harvard Medical School, Boston, for the animals from which these tumors were transplanted.

[3] We wish to thank Dr. W. Sweet, Massachusetts General Hospital, for the opportunity to carry out the studies in these two patients.

Tissues.—The concentration of Rb^{86} in the tissues is shown in Table 4. There is a marked difference between uptakes by normal brain (0.0009–0.0003 per cent/gm) and the tumor tissue (0.0060–0.0058 per cent/gm). In the surgical biopsy specimens obtained about 3½ hours after administration of the Rb^{86}, the tumor demonstrated 6.7 times as much radioactivity per gram as did the normal brain. In the necropsy specimens obtained an hour later, this ratio rose to 19.3. This increase in ratio was related to the more rapid clearance of the isotope from the brain; the concentration in the tumor changed very little during the interval.

CASE 2.—On January 10, 1952, a second patient (T. F.)[4] was operated upon for further resection of a glioblastoma. A solution of Rb_2CO_3 containing 100 μc. of Rb^{86} was injected intravenously after the surgical procedure was under way. Specimens of the glioblastoma and of normal brain were provided at random intervals until the operation was completed.

The uptake of Rb^{86} by the normal brain tissue had already reached a maximum at the time of the 24-minute sampling (Table 5). The concentration in the glioblastoma, however, more than tripled during the next 14 minutes. The ratio of uptake by tumor:brain increased from 1.7:1 at 24 minutes to 6.0:1 at 98 minutes. In view of the apparent more rapid clearing of Rb^{86} from the brain, it is likely that the tumor:brain ratio would have continued to increase.

TABLE 3

UPTAKE OF Rb^{86} AND Cs^{134} BY NEUROGENIC NEOPLASMS AND OTHER TISSUES OF MICE

(Per cent of dose/gm)

	Astrocytoma (C3H mice)				Neuroblastoma (ABC mice)			
	Rb^{86}		Cs^{134}		Rb^{86}		Cs^{134}	
Tissue	4 hrs.	24 hrs.	4 hrs.	24 hrs.	4 hrs.	24 hrs.	4 hrs.	24 hrs.
Tumor	1.82	2.65	0.34	0.69	0.94	1.14	0.21	0.32
Brain	0.18	0.79	0.03	0.15	0.25	0.43	0.13	0.28
Pancreas	2.78	3.43	1.54	1.42	3.79	1.59	1.28	2.86
Liver	2.78	2.72	0.64	0.56	3.49	1.37	0.43	1.45
Muscle	1.38	2.40	0.37	0.81	2.12	1.33	0.36	1.30
Tumor:brain ratio	10.1	3.4	11.3	4.6	3.8	2.7	1.6	1.1

TABLE 4

DISTRIBUTION OF Rb^{86} IN HUMAN BIOPSY AND NECROPSY TISSUE SPECIMENS

Source	Tissue	Time interval	Per cent total dose/gm
Biopsy	Normal brain	3 hrs. 7 min.	0.0009
	Brain tumor	3 " 31 "	0.0060
	Muscle	3 " 37 "	0.0056
	Whole blood	3 " 40 "	0.0004
Necropsy	Normal brain	4 hrs. 38 min.	0.0003
	Brain tumor (metas.)	"	0.0058
	Invaded lymph node	"	0.0053
	Esophageal carcinoma	"	0.0036
	Liver	"	0.0098
	Heart	"	0.0091
	Lung	"	0.0069
	Kidney	"	0.0053
	Muscle	"	0.0040
	Testis	"	0.0038
	Scalp	"	0.0031
	Adrenal cortex	"	0.0029
	Dura	"	0.0006

TABLE 5

DISTRIBUTION OF Rb^{86} IN A GLIOBLASTOMA AND NORMAL BRAIN TISSUE

(Per cent of dose/gm)

Tissue	24 min.	38 min.	60 min.	98 min.
Normal brain	0.007	0.006	0.004	0.0015
Glioblastoma	0.004	0.014	0.014	0.009
Ratio tumor:brain	1.7:1	2.3:1	3.5:1	6.0:1

The intracranial and retroperitoneal metastases showed similar uptakes of Rb^{86}. These differed, however, from the concentration in the esophageal lesion. The discrepancy is possibly due to the heterogeneous nature of the latter specimen, which contained both normal and neoplastic tissue.

The greatest uptake of rubidium was demonstrated in the liver, which contained 0.0098 per cent of the total dose per gram. Concentrations of Rb^{86} in the remaining tissues, in decreasing order were: heart, lung, brain tumor (metastatic), invaded lymph node, kidney, muscle, testis, esophageal carcinoma, scalp, adrenal cortex, dura, and normal brain. Specimens of pancreas and spleen were unfortunately not obtained.

COMMENTS

These studies demonstrated that more Rb^{86} was concentrated in primary brain tumors and in a metastatic brain tumor of esophageal origin than in normal brain tissue. Carcinomas of the esophagus, stomach, breast, and uterus in humans were previously shown (4) to retain more rubidium than certain normal tissues when natural rubidium was administered parenterally. Our observations of tumor to brain ratios for Rb^{86} uptake of 10.1 in the mouse astrocytoma, 19.3 in the patient with

[4] Distribution of Rb^{86} in the blood of this patient is reported elsewhere (8).

metastatic malignancy to the brain, and 6.0 in the patient with glioblastoma, suggest the feasibility of localizing intracranial neoplasms with this isotope by means of external counting devices. The tumor to brain ratios obtained with rubidium are within the range reported by Erickson *et al.* (1) and Selverstone *et al.* (5, 6) using P^{32} and K^{42}. Erickson *et al.* (1), utilizing P^{32}, obtained tumor to normal brain ratios of 1.6–29 (average, 10) to 1 in eleven patients with glioblastoma multiforme. Using P^{32}, Selverstone *et al.* (5) found tumor to normal brain ratios of 5.5–112 (average, 17) to 1 in nine cases of intracranial tumors. With K^{42}, Selverstone *et al.* (6) reported tumor to normal brain ratios of 20–78 to 1 during the first 6 hours, falling to 4–10 to 1 after 18 hours in eight cases of glioblastoma multiforme. Susen *et al.* (7) and Selverstone *et al.* (6) recently reported the successful use of K^{42} and a directional scintillation counter in the detection of brain neoplasms. The physical half-life of rubidium^{86} of 19.5 days and the gamma ray of 1.08 Mev are desirable physical characteristics for this purpose. Possible interference from Rb^{86} in the scalp and its muscles may be minimized by placing the Geiger-Müller tube or scintillation counter at a distance of approximately 10 cm. from the scalp, thus invoking the inverse square law (6).

Distribution of Rb^{86} in the tissues of mice is in good accord with that found in the guinea pig and dog (2). In each of these species, uptake per gram, expressed as the percentage of the dose administered, was greatest in the pancreas, followed closely by spleen and liver. Although a lesser concentration is found in muscle, the deposit of Rb^{86} in the entire muscle mass is very great. In the patient with esophageal carcinoma (Case 1), the entire muscle mass was estimated to contain 70 per cent of the administered dose (muscle mass calculated as 30 per cent of body wt.). These results are consistent with previous observations by Mendel and Closson (3).

SUMMARY

1. Brain tumors in mice and humans were shown to have taken up from 6 to 19.3 times more Rb^{86} per gram than did normal brain tissue. These ratios of concentration are sufficiently great to warrant the use of this isotope in attempted localization of intracranial neoplasms by means of external counting devices.

2. The pancreas demonstrates the highest concentration of administered Rb^{86}, followed closely by liver and spleen. The absolute majority of the isotope is distributed to the entire skeletal muscle of the body.

3. Toxicity studies in mice demonstrated that rubidium has a profound effect upon musculature. Overdosage produces generalized spastic paralysis leading to respiratory failure. The MLD_{50} for mice is 0.65 mg. Rb_2CO_3 per gram of body weight.

4. Doses of Rb^{86} adequate for localization studies in man are well below the range of toxicity.

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Effects of Combinations of Antileukemic Agents on an Acute Lymphocytic Leukemia of Mice*

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Two major obstacles prevent the successful treatment of neoplastic diseases by direct attack on the cancerous cell. First, chemical or physical agents which selectively destroy neoplastic cells without also seriously impairing the function of normal cells are unknown at present, although folic antagonists, in certain experimental leukemias of the mouse, exhibit a striking selectivity of action. Second, the development of variant forms of cancer cells which are resistant to and even dependent upon the specific chemotherapeutic agent used in therapy results in eventual failure of treatment. The development of such resistant and dependent variant cells has been described in the experimental leukemias of mice (4, 13, 16); it is reasonable to assume that similar transformations occur in other neoplasms as well.

The discovery of more effective anticancer agents, which at present seems improbable because of the qualitative similarities of normal and cancer cells, will not eliminate the problem of development of resistance and dependence of cancer cells. It is known, from experimental tests, that the transformations to resistance and to dependence which occur rather generally among experimental leukemias arise as spontaneous mutations which are stable, irreversible, and heritable (13, 14). The anticancer agent acts in selecting out these variant forms. Thus, more effective anticancer agents will be in all probability more effective in selecting spontaneous mutations occurring in the population of cancerous cells.

Extensive genetic investigations of drug resistance in bacteria show that a variety of physical and chemical agents increases mutation rates. There is no known method for decreasing these mutation rates, and it is unlikely that the host can alter the process of spontaneous mutation. The approach to resistance and dependence, therefore, would appear to be in attempting to control or suppress the process of selection of these spontaneously occurring transformations. It was with this objective in mind that the series of experiments reported here was undertaken.

The principle of combined therapy with two or more chemotherapeutic agents, acting independently, has been used successfully in infectious diseases, particularly in the treatment of tuberculosis. If the frequency of mutation to resistance of a cell, bacterial or cancerous, to drug A is 1×10^{-6} and a frequency of mutation to drug B is 1×10^{-5}, only one cell in 10^{11} will simultaneously develop both mutations. Thus, doubly resistant mutants have a negligible probability of emerging from a susceptible tumor or bacterial population in the presence of two or more effective chemical or physical agents which exhibit different mechanisms of action.

Several studies have been made using combinations of anticancer agents. Slight but consistent synergistic action of urethan and nitrogen mustard (HN_2) was shown by Skipper *et al.* (20), using a lymphocytic leukemia (AK4). Goldin *et al.* (8) showed antileukemic synergism in various combinations of 8-azaguanine, alpha-peltatin, and the 4-amino substituted folic antagonist, aminopterin; the most effective combination was of the two antimetabolites, 8-azaguanine and aminopterin. Even with this combination, increase in survival time of leukemic mice was not striking. Shapiro and Gellhorn (19) reported that the inhibition of the growth of adenocarcinoma 755 in C57BL mice was greater in combinations of 8-azaguanine with desoxypyridoxine, folic acid, 7-methylfolic acid, or vitamin B_{12} than with 8-azaguanine alone, although, of the compounds used in combination with the guanine analog, only desoxypyridoxine gave indication of some inhibition. In a preliminary note Emerson, Wurtz, and Zanetti (6) reported that cortisone (compound E), when administered to riboflavin-deficient mice, caused a

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rapid regression of well-established lymphosarcomatous grafts, whereas cortisone alone, or other adenocorticosteroids in combination with the avitaminosis, were ineffective.

Of the antileukemic compounds used in the present study, 4-amino-N^{10}-methylpteroylglutamic acid (A-methopterin) is the most effective in increasing survival time in mice with acute lymphocytic leukemia (3, 13). It has also been used effectively in developing resistant and dependent leukemic cells and resistance in *Streptococcus faecalis* (5). The guanine analog, 8-azaguanine (5-amino-7-hydroxy-1*H*-*v*-triazolo(*d*)pyrimidine) has also been relatively effective in inhibiting growth of leukemic cells in certain leukemic lines (7, 10, 11) but has been inactive against other leukemias (3, 22). This compound, likewise, has been used as an effective selective agent in developing variant leukemic cells showing resistance or dependence (14, 18). It has been shown by use of the resistant variants developed that these two antimetabolites exert their antileukemic effects through different mechanisms. Alpha-peltatin (9) and 2,4,6-triethyleneimine-*s*-triazine (TEM) (2) have also been shown to possess some antileukemic activity.

MATERIALS AND METHODS

The acute lymphocytic leukemia, L 1210 (18), which arose in a DBA strain mouse of subline 212 (DBA/2) and is carried in transplant within this subline, was used as test material. This leukemia has been transferred by a standard dose of cells (8×10^5) (13) into more than 3,000 mice of the DBA/2 strain and into more than 500 BDF_1 mice (♀ C57BL × ♂ DBA). Leukemia has resulted in all cases with no regressions. Leukemic cells, obtained from the lymphomatous tumor mass produced in subcutaneous transfer, were diluted in Locke's solution and inoculated either intraperitoneally or subcutaneously into mice of both sexes weighing 18–20 gm. The tests reported here were accomplished with leukemic cell preparations obtained between the 100th and 150th transfer generation of this leukemia.

A-methopterin[1] was dissolved routinely in distilled water and injected intraperitoneally in a total volume equivalent to 1 per cent of the body weight. 8-Azaguanine was dissolved in 0.1 N NaOH and diluted with distilled water to 0.01 N NaOH. The pH of the resulting solution was approximately 8. Injections were given subcutaneously at a total volume equivalent to 5 per cent of the body weight. Alpha-peltatin was dissolved in 1 N NaOH and then diluted to the concentration used with distilled water. TEM was dissolved in distilled water. Both latter compounds were injected intraperitoneally in a total volume of solution representing 1 per cent of body weight.

8-Azaguanine was given daily, beginning 24 hours after inoculation of leukemic cells. A-methopterin was given every other day, beginning 48 hours after inoculation of leukemic cells. TEM and alpha-peltatin were given every other day, the first injection beginning at 72 hours postinoculation of leukemia. Exceptions to this schedule were made in the experiments of Series 3 (see footnote of Table 3).

Tissues for histologic study were fixed in Tellyesniczky's fluid[2] and stained with hematoxylin and eosin.

RESULTS

Series 1.—Table 1 shows effects of various substances, given either singly or simultaneously, on survival time of mice bearing intraperitoneal transfers of leukemic cells. The dosage schedules for the two most effective compounds, 8-azaguanine and A-methopterin, are within the effective antileukemic range but lower than the maximum tolerated dose. Little or no loss in weight at 7 days was observed in mice receiving these compounds, singly, within the dosage schedules employed; increases in dosage further increased survival time. The dosage range for A-methopterin varied from 3 mg/kg × 4 (75 per cent increase in survival time) to 3 mg/kg × 12 (246 per cent increase in survival time). The dosage range for 8-azaguanine

[1] A-methopterin, 8-azaguanine, and TEM were kindly supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y. Dr. Joseph Leiter of this Institute kindly furnished the alpha-peltatin used in these experiments.

[2] Guyer, M. F. Animal Micrology, p. 214. 3d ed. Chicago: Univ. of Chicago Press, 1934.

NOTES TO TABLE 1

* Total dose represents maximum total dose given in an individual experiment. Mice dying relatively early did not receive maximum total dose. Antileukemic compounds given intermittently after regular schedule in combination groups of Experiments 2, 3, 4, and 5 when condition of animals permitted.

† Am = A-methopterin, 8 Ag = 8-Azaguanine, TEM = Triethylene melamine, AP = Alpha-peltatin.

‡ Reinoculated at 149 days with leukemia L 1210. Dead from leukemia at 11 days.

§ Mice given combination of Am, 8 Ag, and TEM, simultaneously, died from symptoms of toxicity at 9 days.

Injections of Am and 8 Ag continued intermittently on eight animals which lived beyond 36 days.

‖ Two reinoculated at 149 days with leukemia L 1210. Dead at 11 days.

** Three reinoculated with leukemia L 1210 at 180 days. All dead, leukemic, at 10 days.

†† No histologic evidence of leukemia at 90 days in these mice.

‡‡ Survival time based on thirteen survivors.

TABLE 1

Effect of Several Antileukemic Agents, Given Singly or Simultaneously in Combination, on Survival Time of L 1210 Leukemic Mice

(Intraperitoneal transfer of leukemic cells)

Experiment	Strain of mice	No. mice	Dosage (mg/kg) Daily	Dosage (mg/kg) Total*	Survival time in days (range)	Percentage increase in survival	Remarks
1. Control	DBA/2	10	None		8.8 (8–9)		
Am†	"	10	3	27	20.2 (19–22)	129.5	
8 Ag†	"	10	75	750	14.1 (13–16)	60.2	
Am+8 Ag	"	15	3 75	27 750	37.6 (21–76)	327.3	4 dead of leukemia at 71, 71, 72, and 76 days
2. Control	BDF_1	10	None		10.6 (8–14)		
Am	"	10	3	36	36.7 (21–37)	246.2	1 90-day survivor‡
8 Ag	"	8	75	975	16.0 (11–21)	50.9	
TEM†	"	10	0.6	2.4	13.7 (11–15)	30	
Am+8 Ag§	"	11	3 75	45# 1,725	49.8 (17–90)	369.8	1 dead, leukemic, at 64 days 2 90-day survivors‖ 1 dead, toxic symptoms, at 17 days
3. Control	BDF_1	10	None		10.1 (9–12)		
Am	"	10	3	30	31.6 (26–40)	212.9	
8 Ag	"	10	75	900	17.3 (16–19)	71.3	
Am+8 Ag	"	11	2 50	36 1,000	42.9 (29–86)	324.8	2 dead, leukemic, at 83 and 86 days
Am+8 Ag	"	10	3 75	54 1,650	58.9 (21–90)	483.1	1 dead, negative, at 141 days 4 dead, leukemic, at 79, 81, and 82 days 3 dead, at 21 days, with symptoms of toxicity
4. Control	BDF_1	10	None		9.4 (8–12)		
Am	"	8	3	24	20.3 (17–23)	116	
8 Ag	"	10	75	600	14.8 (13–16)	58	
TEM	"	10	0.3	1.2	11.4 (7–13)	21.3	
Am+8 Ag	"	10	3 75	24 900	37.5 (16–90)	298.9	1 dead, negative, at 107 days 1 dead, toxic symptoms, at 7 days
Am+8 Ag+TEM	"	10	3 75 0.3	27 975 1.2	26.2 (9–44)	178.7	4 dead, toxic symptoms, at 9, 12, 14, and 15 days
5. Control	BDF_1	10	None		10.1 (9–12)		
Am	"	10	3	24	20.3 (17–23)	100.9	
8 Ag	"	10	75	750	17.1 (15–19)	71	
TEM	"	10	0.75	3.0	12.4 (11–14)	23	
Am+8 Ag	"		3 75	66 2,025	65.6 (40–90)	549.5	3 lived to 180 days** 2 killed at 90 days for tissue††
Am+8 Ag+TEM	"	11	3 75 0.75	66 2,025 3.75	55.5 (7–90)	449.5	5 leukemic deaths at 61, 61, 89, 96, and 104 days 1 killed at 90 days for tissue†† 3 dead, toxic symptoms, at 7, 10, and 13 days
6. Control	DBA/2	10	None		9.7 (9–11)		
Am	"	10	3	12	17.0 (16–18)	75.3	
8 Ag	"	10	75	600	13.4 (11–15)	38.1	
AP	"	10	0.5	2.0	10.4 (9–13)		
Am+8 Ag	"	10	3 75	12 600	21.1 (19–25)	117.5	
Am+AP†	"	10	3 0.5	12 2.0	18.8 (17–21)	93.8	
Am+8 Ag+AP	"	10	3 75 0.5	12 600 2.0	23.7 (20–28)	144.3	
7. Control	BDF_1	10	None		11.5 (11–12)		
Am	"	15	3	18	18.6 (16–21)	61.7	
8 Ag	"	15	75	750	13.6 (12–16)	18.3	
Am+8 Ag+AP	"	20	3 75 0.5	18 750 1.5	34.3 (24–90)‡‡	198.3	1 90-day survivor 7 dead, toxic symptoms, at 14 days

varied from 75 mg/kg × 8 (58 per cent increase in survival time) to 75 mg/kg × 13 (51 per cent increase in survival time). It may be seen in Experiments 1, 6, and 7 that a striking potentiation of antileukemic effect was obtained upon simultaneous administration, particularly of the two antimetabolites, A-methopterin and 8-azaguanine. In the remaining experiments, where injections were continued intermittently in the combination–therapy groups, increases in survival time up to 549 per cent were obtained. Twelve mice in the combination–therapy group survived for 90 days or longer. Of these, three were killed for pathologic study of the tissues, two died with generalized leukemia at 96 and 104 days, one died without signs of leukemia at 141 days, and the remaining six were reinoculated with standard doses of leukemic cells of leukemia L 1210. All six of the reinoculated 90-day survivors died with generalized leukemia at 10 and 11 days. A decided increase in symptoms of drug toxicity appeared in mice receiving combinations of drugs, particularly in mice receiving TEM and alpha-peltatin in addition to the two antimetabolites used.

Series 2.—The effect on survival time of antileukemic agents given individually or in combination is not particularly striking in mice bearing subcutaneous transfers of leukemic cells (Table 2). Nevertheless, potentiation of antileukemic activity may be seen in all experiments except Exp. 11.

In Exp. 12, a different subline of leukemia L 1210 was used as the source of leukemic cells. A virus-like contaminant has been described in this subline of leukemia L 1210, which strikingly reduced body weight, caused a moderate leuko-

TABLE 2

EFFECT OF ANTILEUKEMIC AGENTS, GIVEN SINGLY OR SIMULTANEOUSLY IN COMBINATION, ON SURVIVAL TIME OF L 1210 LEUKEMIC MICE
(Subcutaneous transfer of leukemic cells)

Experiment	Strain of mice	No. mice	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Survival time in days (range)	Percentage increase in survival	Remarks
8. Control	DBA/2	13	None		12.9 (11–15)		
Am*	"	10	3	12	18.3 (16–22)	42	
8 Ag*	"	10	75	750	16.9 (14–23)	31	
AP*	"	10	0.5	2.0	15.0 (14–17)	16.2	
Am+8 Ag+AP			3	12			
	"	16	75	750	29.3 (25–35)	127.1	
			0.5	2.0			
9. Control	DBA/2	5	None		14.0 (13–15)		
Am	"	5	3	12	21.4 (18–23)	52.8	
8 Ag	"	5	50	400	15.8 (14–18)	12.8	
Am+8 Ag			3	12			
	"	8	50	400	21.7 (20–24)	55.5	
Am+8 Ag+AP	"	8	Same		36.7 (23–90)	162.6	1 90-day survivor†
10. Control	DBA/2	10	None		13.2 (13–14)		
Am	"	10	3	12	19.1 (17–21)	44.7	
8 Ag	"	7	75	600	15.0 (14–16)	14.4	
AP	"	10	0.15	2.0	14.6 (12–18)	10.6	
Am+8 Ag	"	15	Same		23.7 (21–29)	79.6	1 dead 14 days, toxic symptoms
Am+AP	"	15	Same		22.1 (20–27)	67.5	1 dead 14 days, " "
Am+8 Ag+AP	"	15	Same		29.0 (22–90)	119.7	1 90-day survivor†
11. Control	BDF_1	8	None		14.3 (13–16)		
Am	"	10	3	12	25.3 (23–28)	77	
8 Ag	"	8	75	600	21.8 (17–24)	52.5	
TEM*	"	8	0.75	3.0	19.1 (17–20)	33.6	
Am+8 Ag	"	10	Same		30.1 (28–35)	110.5	
Am+8 Ag+TEM	"	10	Same		32.0 (27–39)	123.8	3 dead, toxic symptoms at 15, 15, and 17 days
12. Control	DBA/2	8	None		19.6 (19–22)		
Am‡	"	8	3	12	24.6 (23–27)	25.5	
8 Ag	"	10	50	400	21.4 (21–24)	9.2	
AP	"	7	0.5	1.5	16.4 (12–24)		
Am+8 Ag	"	10	Same		45.9 (27–90)	134.2	3 90-day survivors†
Am+8 Ag+AP	"	10	Same		71.0 (18–90)	262.7	4 90-day survivors† 3 dead of toxic symptoms at 8, 12, and 12 days

* Am = A-methopterin, 8 Ag = 8-Azaguanine, AP = Alpha-peltatin, TEM = Triethylene melamine.

† All 90-day survivors reinoculated with leukemia L 1210. All dead at 10 and 11 days with generalized leukemia.

‡ Subline of leukemia L 1210, contaminated with a virus-like agent (17) used in this experiment. Note increase in survival time of control group over controls in noninfected line.

cytosis (due principally to an increase in granulocytes), increased the mean age at death in mice bearing subcutaneous transfers of leukemic cells, and seriously injured normal lymphocytes particularly in the spleen, thymus, and lymph nodes; it likewise injured infiltrating leukemic lymphocytes (17). The survival time of mice bearing subcutaneous transfer of this particular subline of leukemia was strikingly increased by use of a combination of A-methopterin and 8-azaguanine and with the two antimetabolites plus alpha-peltatin, although, in the latter group, three mice died early without leukemia. Seven 90-day survivors were found in these two groups, and all were found to be sensitive to standard doses of leukemia L 1210 when reinoculated, dying of leukemia at 10 and 11 days.

Further tests, using the control subline of leukemia L 1210, and introducing the nonlethal, virus-like contaminant, failed to show the striking modification of the leukemic process in mice given combinations of these same antileukemic agents.

Series 3.—It may be seen by reference to Table 3 that the most effective antileukemic regimen, in the particular leukemia used, is the simultaneous administration of the two drugs. When a series of injections of either A-methopterin or 8-azaguanine is given first to leukemic DBA or BDF_1 mice followed immediately by a series of injections of the other compound, increases in survival time greater than with either agent also are obtained. It seems unimportant which compound is administered first. When the two compounds are given simultaneously, however, within the dosage range used, an increase in survival time of 273 per cent was obtained in DBA mice and 325 per cent in BDF_1 mice. Thirteen mice (23 per cent) survived 90 days or longer free of leukemia in the series receiving antimetabolites simultaneously, but none out of 114 mice receiving the same dosage of the two compounds, given singly, survived. Of the seventeen 90-day survivors, one died without leukemia at 141 days of age, and the remaining twelve were reinoculated at 90–150 days. All were found to be susceptible to leukemic cells of L 1210 and died from generalized leukemia at 8–10 days following inoculation. A somewhat greater loss in weight occurred in these animals receiving both antimetabolites simultaneously and six mice (4 per cent) died of toxic symptoms at 9–12 days without evidence, grossly, of leukemia.

90-Day survivors.—Thirty-five out of 320 (11 per cent) DBA/2 and BDF_1 leukemic mice given combinations of A-methopterin and 8-azaguanine, or these two compounds plus either alpha-peltatin or TEM, simultaneously, survived beyond 90 days. In contrast, only one in 418 leukemic mice given these antileukemic compounds either singly or singly in combination (Series 3) survived to 90 days (Experiment 2, Table 1). None of 186 untreated leukemic mice survived beyond sixteen days. In addition, many mice in the combination group died of leukemia up to the 90-day period (see legend, Chart 1).

Twenty-four 90-day survivors (90–180 days) were reinoculated intraperitoneally with standard doses of L 1210 leukemic cells. All 24 were found to be susceptible to reinoculation and died from generalized leukemia at 8–11 days; four died without evidence of leukemia at 90, 107, 140, and 141 days, respectively; three were sacrificed for histologic examination at 90 days; two died, leukemic, at 96 and 104 days, and two appeared without leukemic symptoms at 90 days. No evidence of leukemia was found in the mice sacrificed for histologic studies.[3]

DISCUSSION

The present set of experiments was undertaken in an attempt to control or suppress the selection of resistant and dependent leukemic cells which arise, invariably, when large populations of these cells are exposed to certain inhibitory agents. The two most effective compounds, A-methopterin and 8-azaguanine, are known to act as selective agents in the isolation of resistant and dependent variant leukemic cells in the acute lymphocytic leukemia L 1210 employed in these studies. The compounds also are known to act independently of each other.

Striking prolongation of survival time in leukemic mice given various dosage levels of A-methopterin and 8-azaguanine simultaneously were obtained. Potentiation of antileukemic effect is evident in those experiments where the compounds were administered for a period of time and then stopped. Alpha-peltatin and TEM, given in conjunction with the two antimetabolites, did not appreciably enhance this potentiation, probably because of added systemic toxicity.

The obvious mechanism of action of the combination utilized is the selective destruction of sensitive leukemic cells and the suppression of doubly resistant mutants. Animals which become leukemic beyond the 45-day period (see legend, Chart 1) in all probability represent animals in which sensitive cells were not completely destroyed or in which doubly resistant mutant leukemic cells arose. Since prolonged administration

[3] Dr. Thelma B. Dunn, Laboratory of Pathology, has kindly done the histologic study of these tissues.

of A-methopterin and 8-azaguanine was attempted to only a limited degree, because of host toxicity, it is impossible to distinguish the two possibilities. Animals which survived 90 days or beyond in all probability represent cases in which all, or most, sensitive leukemic cells were killed and doubly resistant forms completely suppressed.

Numerous reports in the literature have disclosed survival of treated leukemic mice (acute lymphocytic leukemia or lymphosarcoma) beyond 60 or 90 days. Some such survivors were also found among untreated control leukemic mice. These, in all probability, represent a condition of immunologic response on the part of the host resulting from genetic differences between host and tumor. They are usually characteristic of tumors

TABLE 3

EFFECT OF A-METHOPTERIN AND 8-AZAGUANINE GIVEN IN COMBINATION, EITHER SINGLY OR SIMULTANEOUSLY, ON SURVIVAL TIME OF TEST MICE BEARING ACUTE LYMPHOCYTIC LEUKEMIA L 1210

Experiment*	Strain of mice	No. mice	Dosage (mg/kg) Am	Dosage (mg/kg) 8 Ag	Survival time in days (range)	Percentage increase in survival	Wt. changes†	Remarks
13. Control	DBA/2	10	None		8.1 (8–9)		+1.0 gm	
Am–8 Ag	"	10	3×5	75×9	17.0 (12–22)	112.5	−1.7 gm	
8 Ag–Am		10	3×5	75×9	18.6 (18–19)	129.6	−0.4 gm	
Am+8 Ag (Simult.)	"	10	3×5	75×9	36.5 (20–90)	350.0	−1.2 gm	2 90-day survivors‡
14. Control	DBA/2	16	None		7.6 (7–9)		+2.2 gm	
Am–8 Ag	"	9	3×4	75×8	19.2 (18–20)	152.7	+0.6 gm	
8 Ag–Am	"	9	3×4	75×8	20.1 (18–22)	164.5	+0.1 gm	
Am+8 Ag (Simult.)	"	9	3×4	75×8	28.4 (19–90)	273.6	−0.4 gm	1 90-day survivor§
15. Control	DBA/2	16	None		8.1 (7–10)		+2.6 gm	
Am–8 Ag	"	8	3×4	75×8	20.0 (19–21)	146.9	+0.2 gm	
8 Ag–Am	"	8	3×4	75×8	16.1 (15–17)	98.7	+0.2 gm	
Am+8 Ag (Simult.)	"	16	3×4	75×8	25.7 (11–90)	217.3	−0.7 gm	2 died at 9 days 2 90-day survivors#
1) Control	DBA/2	42			7.9±0.01			
Totals 2) Am–8 Ag and 8 Ag–Am	"	54	(See above)		18.5±0.33	134.2	Diff. (1) and (3) = 21.6±4.0 P<0.001	
3) Am+8 Ag (Simult.)	"	35			29.5±4.0	273.4	Diff. (2) and (3) = 11.0±4.1 P<0.01	
		131						
16. Control	BDF_1	10	None		9.7 (8–12)		+1.2 gm	
Am–8 Ag	"	10	3×5	75×9	20.0 (11–29)	106.2	−1.5 gm	
8 Ag–Am	"	10	3×5	75×9	19.6 (16–25)	102.1	−0.6 gm	
Am+8 Ag (Simult.)	"	10	3×5	75×9	52.4 (24–90)	440.2	−1.6 gm	4 90-day survivors‖
17. Control	BDF_1	10	None		10.6 (9–12)		+0.3 gm	
Am–8 Ag	"	10	3×4	75×8	22.2 (19–25)	109.4	−0.6 gm	
8 Ag–Am	"	10	3×4	75×8	20.5 (16–30)	94.2	+0.7 gm	
Am+8 Ag (Simult.)	"	10	3×4	75×8	36.7 (20–90)	246.2	−1.0 gm	2 90-day survivors**
18. Control	BDF_1	10	None		10.5 (9–14)		+1.5 gm	
Am–8 Ag	"	10	3×4	75×8	25.0 (21–34)	142.8	−0.5 gm	
8 Ag–Am	"	10	3×4	75×8	17.4 (13–20)	70.0	−0.9 gm	
Am+8 Ag (Simult.)	"	10	3×4	75×8	41.0 (22–90)	290.5	−1.4 gm	4 died with toxic symptoms at 11 and 12 days; 2 90-day survivors††
1) Control	BDF_1	30			10.26±0.23			
Totals 2) Am–8 Ag and 8 Ag–Am	"	60	(See above)		20.78±0.63	101.9	Diff. (1) and (3) = 33.5±5.6 P<0.001	
3) Am+8 Ag (Simult.)	"	30			43.8 ±5.6	324.8	Diff. (2) and (3) = 23.02±5.7 P<0.001	
		120						

* Am–8 Ag = A-methopterin given first every other day beginning at 48 hrs., followed by series of injections of 8-azaguanine given daily; 8 Ag–Am = 8-Azaguanine series of injections given first, beginning at 24 hours followed by A-methopterin; Am+8 Ag (Simult.) = Injections of both compounds given simultaneously, 8-azaguanine daily beginning at 24 hrs., A-methopterin every other day beginning at 48 hours. No injections given after the 18th post-inoculation day.

† Weight changes at 7 days following inoculation of leukemic cells.

‡ Reinoculated L 1210 at 90 days; 1 dead 8 days, 1 dead 10 days; both leukemic.

§ Reinoculated at 105 days with leukemia L 1210. Dead, leukemic, at 8 days.

Both alive at 90 days. No evidence of leukemia.

‖ All 4 90-day survivors reinoculated at 90 days. All dead, leukemic, at 10 days.

** Both reinoculated at 150 days with leukemia L 1210. Both dead, leukemic, at 8 days.

†† One animal died without leukemia at 141 days. Other, inoculated at 150 days with L 1210. Dead, leukemic, at 8 days.

transplanted into sublines of inbred mice different from that of the origin of tumor, or characteristic of tumors which have been carried in transplant for many years, thus allowing genetic mutations to occur in the strain of mice or in the tumor. This is probably the explanation for the peculiar immunologic response of lymphosarcoma 6 C3H-ED to colchicine (1) and to riboflavin deficiency (21). Complete regression of established implants of this tumor in C3H strain mice occurred. Reimplantation, after 60 days or longer, resulted in complete failure of growth of the specific 6 C3H-ED lymphosarcomatous cells. All of the DBA/2 or BDF_1 mice in this study which survived the combination treatment, 90 days or longer, and which were reinoculated with the standard dose of L 1210 leukemia, succumbed to a generalized leukemia within the expected time.

The data in Series 3 provide a clear rationale for the use of two or more chemotherapeutic agents acting independently. Theoretically, double mutants to resistance should be obtained easily by selection, first with one agent and then with another agent, acting independently. Doubly resistant mutants, on the other hand, have a negligible probability of arising in the presence of effective concentration of the chemotherapeutic agents. Whether or not this is the explanation for the striking prolongation of survival time in the present experiments remains to be determined.

Shapiro and Gellhorn (18) observed enhanced inhibition of growth of a mammary carcinoma in C57BL mice when pteroylglutamic acid, its 7-methyl analog, a weak folic antagonist, or vitamin B_{12} was given in conjunction with 8-azaguanine. The explanation given for the folic acid effect was that one of its degradation products inhibited a liver enzyme system which deaminates 8-azaguanine, the resulting 8-azaxanthine being inactive as a carcinostatic agent. This appears not to be an explanation for the enhanced inhibition noted here. Enhancement of inhibition was not noted in our experiments[4] when the time relations were changed so that A-methopterin (or synthetic folic acid) was given prior to the 8-azaguanine.

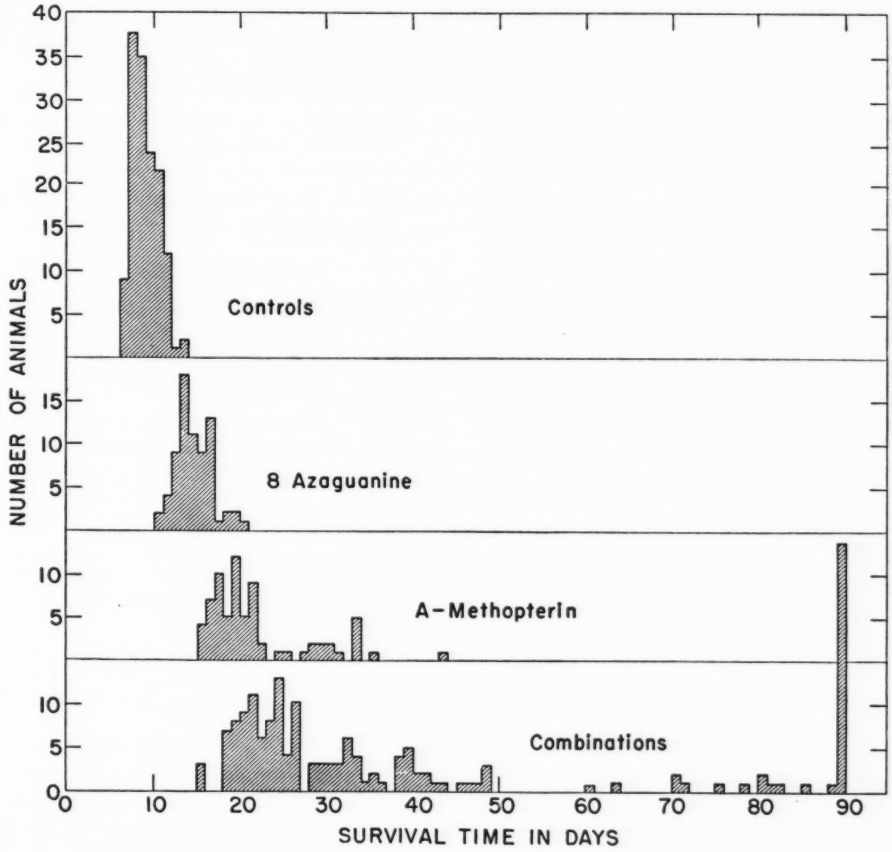

CHART 1.—Distribution of leukemic deaths in DBA/2 and BDF_1 mice. Chart includes only those mice given intraperitoneal inoculations of a standard dose (8×10^5 cells) of leukemia L-1210. Combination group includes only those mice given antileukemic compounds simultaneously.

Deaths from systemic drug toxicity are not included in the combination group. See text for a discussion of 90-day survivors.

[4] L. W. Law and P. J. Boyle, unpublished observations.

The chemotherapeutic approach presented here, based upon our knowledge of the genetics of resistance and dependence phenomena, appears to offer encouragement. It is expected that more satisfactory results will appear upon development of optimal dosage schedules and discovery of more effective antileukemic agents. It is quite obvious, however, that severe limitations are encountered in any plan of therapy for neoplastic diseases.

SUMMARY

An approach to the problem of chemotherapy of experimental leukemia, based upon knowledge of the mechanism of resistance, is presented.

The effects of several antileukemic compounds on survival time of mice bearing the acute lymphocytic leukemia, L 1210, have been studied. Striking potentiating effects were found in leukemic mice given A-methopterin and 8-azaguanine simultaneously. Alpha-peltatin and triethylene melamine did not significantly influence the effects of the two antimetabolites.

A-methopterin and 8-azaguanine given simultaneously in combination were more effective than the two compounds given singly in combination.

Thirty-five out of 320 mice given combinations of antileukemic compounds simultaneously survived 90 days or longer. These mice were apparently nonleukemic, and all those reinoculated with leukemic cells proved to be susceptible.

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Nicotinamide Content of Some Normal and Malignant Tissues; The Apparent Absence of Niacin in Epidermis*

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In previous investigations by this laboratory it was shown that a polarographically reducible substance, characteristic of the epidermis of mouse and man, was altered chemically or lost when this tissue became malignant (2). Changes of a similar nature were postulated for the malignant transformation of muscle and liver cells of mice. The results given in this report indicate in greater detail the nature of these chemical changes in carcinogenesis.

MATERIALS AND METHODS

Mouse and human epidermis (the latter was obtained from amputated extremities after operation) was removed from the dermis at 50° C. according to the method of Baumberger, Suntzeff, and Cowdry (1). Mouse liver and skeletal muscle and horse cardiac and skeletal muscle were also used for the normal tissues. The following transplantable tumors were employed: squamous-cell carcinomas of different degrees of differentiation, hepatoma,[1] rhabdomyosarcoma,[2] and Sarcoma 37. Samples of normal and malignant tissue were removed quickly from the animals and were placed immediately into various solvents and then extracted. Cardiac and skeletal muscle of the horse was obtained fresh at the slaughter house, immediately frozen with dry ice, and then stored at −20° C. until ready for use. The polarographically reducible and ultraviolet absorbing materials were extracted and purified as previously described (3) or were extracted with acetone.

Polarography was carried out in mixtures of purified dioxane (9) (50 per cent by volume) and sodium citrate-citric acid buffers (50 per cent by volume) with tetrabutylammonium iodide (0.1 M) as the supporting electrolyte (3). More recently, 0.1 M sodium borate has also been employed as the supporting electrolyte (15, 16). A Model DU Beckmann spectrophotometer was used for making the absorption curves. Whatman's No. 1 filter paper (sheets $18\frac{1}{4} \times 22\frac{1}{2}$ inches) was used for the paper partition chromatography. The purified reducible materials, obtained as described above, were dissolved in 2 ml. of alcohol or water and spread along a 12–24-cm. line about 8 cm. from the bottom of the paper. Chromatography was carried out in large glass jars (ascending) or in closed boxes (descending), both of which were saturated with the particular solvent employed. *n*-Butanol saturated with water was the most commonly employed solvent. The ultraviolet (UV) absorbing materials on the papers were traced by means of a UV lamp and the R_F values calculated. The UV absorbing bands were then cut out from the large sheets, eluted with distilled water, and dried at 60° C. in a vacuum oven. The material thus obtained from each UV absorbing band was used for spectrophotometry, polarography, or for the isolation of a particular substance.

RESULTS

Previous studies have shown that the polarographically reducible substance present in normal and methylcholanthrene-treated epidermis differed from that present in methylcholanthrene-induced and transplantable squamous-cell carcinomas. These differences were in the half-wave potentials, specific absorption in the ultra-violet, and in the counter-current distribution patterns in a *n*-butanol-water system (2). The results on the reducible material from mouse and human epidermis and from squamous-cell carcinomas following paper chromatography with *n*-butanol satu-

* This investigation was aided by grants from the Charles F. Kettering Foundation and the Damon Runyon Memorial Fund.

[1] The original tumor was obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

[2] The original tumor was obtained from Dr. E. U. Green, Institute for Cancer Research, Fox Chase, Philadelphia.

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rated with water as a solvent are shown in Table 1. They are the average of a number of analyses for each tissue. The material from epidermis has only one reducible and UV absorbing band with a R_F value signally different from the reducible material of the other tissues. On the other hand, the material from squamous-cell carcinomas has four UV absorbing bands. The top band (R_F value 0.58) is reducible with half-wave potential, absorption maximum, R_F value and ϵ/i_d value significantly different from that of the material of epidermis. which are nearly the same as those of the four tumors and heart. The substances absorbing at 260 mμ did not show pronounced shifts in their maxima with changes in pH (7).

The nature of the reducible and UV absorbing material in muscle.—About 400 gm. of horse skeletal muscle were extracted with the alcohol-ether mixture, as described above, and the purified material chromatographed on fifteen sheets of paper The UV absorbing bands (R_F value of about 0.6) were eluted separately with water, the aqueous solu-

TABLE 1

REDUCIBLE AND ULTRAVIOLET ABSORBING MATERIALS FROM TISSUES EXTRACTED WITH ALCOHOL AND ETHER FOLLOWING PAPER CHROMATOGRAPHY WITH BUTANOL AS A SOLVENT

Tissue	No. bands	R_F	Absorption maximum (mμ) pH 7.0	$E_{\frac{1}{2}}$* vs. S.C.E. pH 5.1–5.2 (v.)	Total absorption / Diffusion current = $\frac{\epsilon(\text{pH } 1.5)\dagger}{i_d(\text{pH } 5.2)}$
Epidermis	1	0.30	275	−1.45	8.0
Squamous-cell carcinomas, hepatoma, and Sarcoma 37	4	(a)0.09	249		
		(b)0.15	260		
		(c)0.30	260		
		(d)0.58	260	−1.34	4.4
Muscle	2	(a)0.16	235		
		(b)0.57	260	−1.35	4.9
Rhabdomyosarcoma	4	(a)0.09	249		
		(b)0.21	260		
		(c)0.36	260		
		(d)0.66	261, 350	−1.33	4.1
Liver	4	(a)0.09	251		
		(b)0.19	260		
		(c)0.40	260		
		(d)0.65	260	−1.33	5.0
Heart	4	(a)0.10	246		
		(b)0.16	236		
		(c)0.44	261		
		(d)0.74	261	−1.34	3.8

* Half-wave potential versus the saturated calomel electrode (S.C.E.).
† Extinction at pH 1.5 divided by the diffusion current, i_d at pH 5.2.

That these two reducible compounds are related has been indicated (2). The material from muscle is different from that of epidermis, whereas the material from the rhabdomyosarcoma has UV absorbing bands similar to those of the squamous-cell carcinomas but without a substance absorbing at 235 mμ. However, about one-half of these tumors have given a substance which also absorbs at 350 mμ and which always appears on the paper with an R_F value the same as that of the reducible substance. Insufficient amounts have been obtained to test its migration with other solvents, but its solubility in ether, water, and alcohol is about the same as that of the reducible substance. The UV absorbing material from cardiac muscle is different from that of skeletal muscle and epidermis, in that four UV absorbing bands were present. Finally, the material from liver and hepatoma also has four UV absorbing bands, the R_F values of tions combined and dried *in vacuo* at 60° C. The reducible material was soluble in alcohol and water and appreciably soluble in ether. Solubility in the latter solvent was used for crystallization of the substance in the following manner: The combined material was extracted 3 times with 10–12-ml. portions of ether, and filtered into a 50-ml. beaker. The ether solution was concentrated to a volume of 8–10 ml. and then poured into a 15-ml. centrifuge tube. The solution was then concentrated by warming it carefully in a beaker of warm water to a volume of about 4 ml., at which concentration white needles came out of solution. The yield was increased by inserting the centrifuge tube into an ice bath. The crystals were then packed by centrifuging at 0° C., washed with a small amount of ether, centrifuged again, and then dried at room temperature. This crystalline material from horse muscle was identified as nicotinamide by the com-

bined technics of x-ray diffraction, electrometric titration, and infrared spectrophotometry.[3]

The UV absorbing material obtained from cardiac and skeletal muscle of R_F value of about 0.16 which absorbs maximally at 235 mμ at pH 7.0, was shown to be creatinine by these characteristics.

Nature of the reducible materials from the other normal tissues (except epidermis) and from the tumors.—The data in Table 1 indicate that the reducible substance (R_F value of about 0.6) from liver, heart, and the tumors is nicotinamide. However, since nicotinic acid and N¹-methylnicotinamide have similar half-wave potentials, absorption maxima and ϵ(pH 1.6)/i_d ratios (Table 3), it is necessary to rule out these two compounds. The R_F values of these pyridine compounds are significantly different from that of nicotinamide with butanol as a solvent (see also [12]). Furthermore, the crystalline material obtained from liver and Sarcoma 37, as described above for muscle, gave melting points to within 3–4 degrees of that of nicotinamide.

Further proof that nicotinamide and not nicotinic acid or N¹-methylnicotinamide was present in the tumors and in liver and heart was obtained by a comparison of the diffusion current ratio in 0.1 M sodium borate and in the dioxane-citrate buffer solution of pH 5.1 to 5.2. These results are shown in Table 2. Nicotinic acid (0.2 and 0.3 mg/2 ml) has a higher diffusion current in the dioxane-citrate buffer than in the sodium borate solution; the ratio is 0.65. N¹-methylnicotinamide at the same concentration has a slightly greater diffusion current in the sodium borate solution; the ratio is 1.15. However, the diffusion current for nicotinamide (at 0.2 and 0.3 mg/2 ml) is 2.6 times greater in sodium borate than it is in the dioxane-citrate buffer. Ratios of this order of magnitude were obtained from the purified material (prior to paper chromatography) from liver and the four tumors. This fact also indicated that the reducible compound in these tissues was nicotinamide.

TABLE 2

RATIO OF DIFFUSION CURRENT, i_d, OF SEVERAL PYRIDINE COMPOUNDS IN 0.1 M SODIUM BORATE AND IN A DIOXANE CITRATE BUFFER SOLUTION OF pH 5.1 TO 5.2

Compound	mg/2 ml	a i_d in 0.1 M sodium borate (μamp.)	b i_d in dioxane citrate buffer (μamp.)	a/b
Nicotinic acid	0.2	1.22	2.00	0.61
" "	0.3	2.00	2.94	0.68
N¹-methyl nicotinamide	0.2	2.28	1.95	1.17
" "	0.3	3.24	2.88	1.12
Nicotinamide	0.2	5.40	2.06	2.67
"	0.3	8.52	3.24	2.63

[3] Authors are deeply indebted to the Physiocochemical Research Division of the Eli Lilly Research Laboratories for these data.

Finally, the nature of the current-voltage curves obtained from the purified material (prior to paper chromatography) from liver, heart, and the tumors in 0.1 M sodium borate was the same as that of nicotinamide. Such a current-voltage curve is shown in Chart 1, and the characteristic double wave of nicotinamide is quite apparent. This double wave probably represents the reduction of this compound to dihydronicotinamide (14). On the other hand, nicotinic acid and N¹-methylnicotinamide give only a single wave following electrolysis in 0.1 M sodium borate.

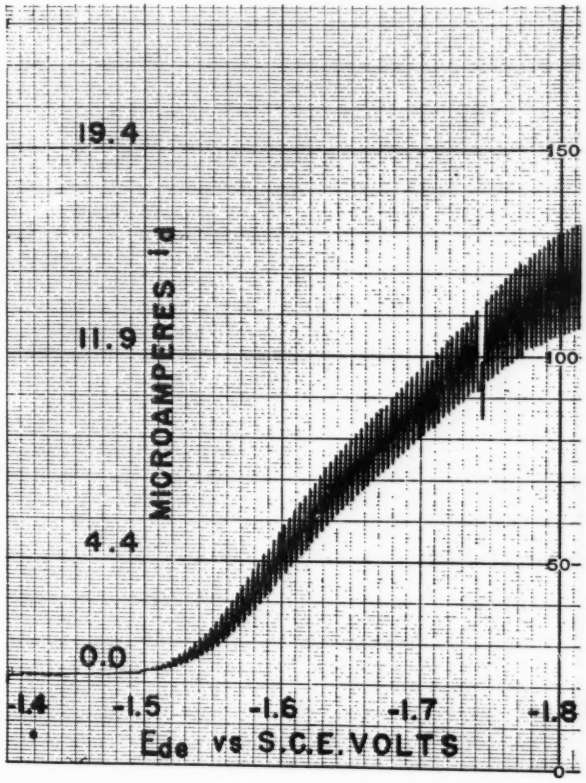

CHART 1.—Polarogram of nicotinamide (0.25 mg/ml) in 0.1 M sodium borate.

Polarography of some other pyridine compounds in dioxane-citrate buffer solutions.—Since the polarographic method is suitable for the quantitative determination of nicotinamide, the half-wave potentials of some other pyridine compounds of biological importance were determined. The data are shown in Table 3. The effects of substituents on the pyridine ring on the half-wave potentials of these reducible compounds offer analytical advantages.

Calibration curves for nicotinamide, nicotinic acid, and N¹-methylnicotinamide are shown in Chart 2. The diffusion currents plotted against the concentrations of these compounds gave straight lines. The polarographic method is ideally suited for the quantitative determination of these and probably other pyridine compounds, since small volumes can be used for electrolysis.

TABLE 3

HALF-WAVE POTENTIALS OF SOME PYRIDINE COMPOUNDS IN DIOXANE-CITRATE BUFFER SOLUTIONS

Name of compound	$E_{\frac{1}{2}}$ vs. S.C.E.* pH 5.1 (v.)	pH 6.6	i_d/C (μamp/mM/l)	Absorption maximum pH 7.0 ($m\mu$)			pH 1.5	$\frac{\epsilon(\text{pH } 1.5)\dagger}{i_d(\text{pH } 5.2)}$
Nicotinic acid	−1.34		2.26		263		261	4.97
Nicotinamide	−1.32		2.52		262		261	4.97
N¹-Methylnicotinamide‡	−1.35		2.67		261.5		262	5.01
Pyridoxamine-HCl	−1.41	−1.54	3.45	219	251	326	293	
Pyridoxal-HCl		−1.68	5.10	220	253	316	289	
Pyridoxine-HCl		−1.58	4.28	221	253	325	291	

* Half-wave potential versus the saturated calomel electrode (S.C.E.).

† Extinction at pH 1.5 divided by the diffusion current, i_d at pH 5.2.

‡ Generously supplied by the Eli Lilly Research Laboratories.

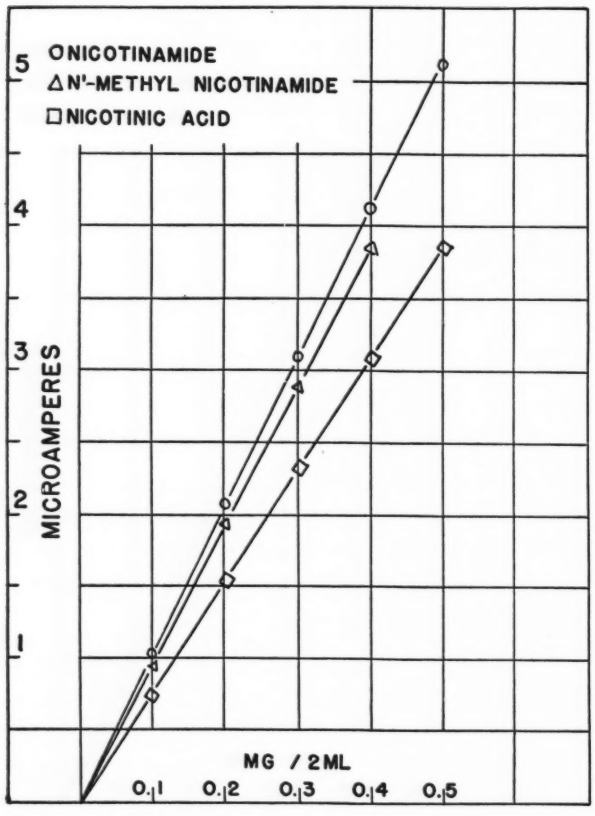

CHART 2.—Calibration curves for nicotinic acid, N¹ methylnicotinamide, and nicotinamide in dioxane and sodium citrate-citric acid buffer solution with tetrabutylammonium iodide. The pH of this solution was 5.1 to 5.2.

The nature of the reducible material from mouse and human epidermis.—The reducible material from epidermis can be purified from paper by the same procedure as that which was used for nicotinamide. A single UV absorbing band obtained from paper, with lutidine as a solvent, was eluted with water and concentrated *in vacuo* at 60° C. until several ml. of water remained. Upon further evaporation at 0 to 4° C., crystals of the polarographically reducible substance appeared. These were washed with cold water and then dried overnight in a vacuum desiccator. These crystals had the half-wave potential, absorption maximum, and ϵ/i_d ratio as indicated in Table 1 for epidermis. The substance melted at 231°–232° with decomposition and had pKá values of 4.0 and 6.04.[3] This crystalline material was treated with 6 N HCl at 105° C. for 24 hours, dried over NaOH until acid-free, and then chromatographed in two dimensions with phenol and lutidine (5). This procedure failed to reveal the presence of ninhydrin reactive substances.

The half-wave potential of the reducible substance from epidermis is in the range of the half-wave potentials of pyridoxamine, pyridoxine, and pyridoxal, but its absorption spectrum is quite different (Table 3). Pyridoxic acid and its lactone are also closely related to pyridoxal, but their properties are different from those of the substance from epidermis. For example, pyridoxic acid is slightly soluble in water and alcohol and insoluble in ether, and it gives the cyanogen bromide test for the pyridine ring (8). Furthermore, pyridoxic acid lactone absorbs maximally near 360 mμ with a shoulder at 250 mμ at pH 7.16 (13). Also, at this pH, pyridoxic acid absorbs maximally at about 310 mμ (13). In contrast, the substance from epidermis absorbs maximally at 275 mμ at pH 7.0. Moreover, the compound characteristic of epidermis is very soluble in water and alcohol and relatively soluble in ether. Furthermore, the substance from epidermis does not give the cyanogen bromide test for the pyridine ring so that the pyridine nitrogen, if present, does not exist in tertiary form or a substituent in position 2 is present.

Also, pyridoxic acid fluoresces blue under ultraviolet light (8), a property not shared by the substance from epidermis.

In the transformation of epidermis to squamous-cell carcinomas by methylcholanthrene, this substance, as such, either disappears or is altered to nicotinamide. In papillomas of mouse skin, the polarographic and UV absorbing properties of the reducible substance of epidermis are altered in such a way that they lie between those of the latter and nicotinamide (Table 4). The data for the papillomas are the average of three samples, two of which were purified on paper with butanol, and indicate that the reducible compound of epidermis has been slightly altered. Nicotinamide was not detectable on the paper chromatograms. In highly differentiated squamous-cell carcinomas (induced or transplantable), there is an abrupt change in the properties of the reducible compound to that of nicotinamide.

TABLE 4

POLAROGRAPHIC AND ABSORPTION CHARACTERISTICS OF THE REDUCIBLE SUBSTANCE OF EPIDERMIS AND PAPILLOMAS OF MICE

Tissue	$E_{\frac{1}{2}}$* vs. S.C.E. pH 5.1 (v.)	Absorption maximum (mμ) pH 7.0	pH 1.5
Epidermis	−1.45	275	267
Papillomas	−1.42	270	265
Carcinomas (Nicotinamide)	−1.35	261	261

* Half-wave potential.

The influence of different solvents on the extraction of nicotinamide.—The effect of various solvents on the amount of nicotinamide extracted from several normal tissues and tumors is shown in Table 5. The amount of this substance extracted from liver with acetone was increased considerably over that obtained with alcohol and ether. Heat was not necessary for the extraction of this substance, since considerable amounts were obtained from muscle and liver by homogenization with acetone, followed by solvent removal at room temperature. The rapidity of the removal of liver and muscle from the animals directly into the acetone or the alcohol-ether mixture did not appreciably influence the amount of nicotinamide extracted. The tumors contained less of this substance than did liver and muscle.

TABLE 5

THE INFLUENCE OF DIFFERENT SOLVENTS ON THE AMOUNT OF NICOTINAMIDE EXTRACTED FROM VARIOUS TISSUES

Tissue	Solvent	No. samples	Nicotinamide (mg/100 gm fresh tissue) (Mean)	(Av.)
Mouse liver	alc.-ether	2	2.1 – 2.3	2.2*
" "	" "	2	4.9 – 6.0	5.5
" "	acetone	2	8.7 –10.0	9.4
" "	"	1	7.92†	
Mouse muscle	alc.-ether	1	1.3*	
" "	" "	3	1.8 – 2.2	2.0
" "	acetone	4	3.2 – 4.4	3.8
" "	"	1	3.2†	
Sarcoma 37	alc.-ether	2	0.8 – 0.9	0.85*
" "	" "	7	1.1 – 2.0	1.73
" "	alc.-acetone	1	1.73	
" "	acetone	1	1.5†	
" "	"	3	1.7 – 3.9‡	2.9
Squamous-cell carcinomas	alc.-ether	4	1.5 – 2.7	1.95
	" "	1	0.55*	
	acetone	1	1.57	
Rhabdomyosarcoma	alc.-ether	5	1.34– 1.8	1.52
"	acetone	1	2.15	
Hepatoma	alc.-ether	5	1.1 – 1.7	1.43
"	" "	1	0.70*	
"	acetone	1	1.45	

* Extracted in absence of O_2.

† Homogenized and extracted at room temperature.

‡ Contained one sample of very small tumors having a high niacin content.

DISCUSSION

In our studies on polarographically reducible substances, we recently postulated that they were characteristic for epidermis, muscle, and liver, and, furthermore, that cleavage of these substances occurred in tumors derived from these tissues (2). The evidence presented in this paper indicates that this is not the case. Only epidermis of the tis-

sues examined has a characteristic reducible substance, which appears to contain a pyridine ring.

Nicotinamide has not been found in epidermis following its extraction with mixtures of alcohol and ether of various compositions, or with acetone or ether alone. Furthermore, this substance has not been found polarographically in epidermis extracted according to the procedure of Chaudhuri and Kodicek (4) which determines the total nicotinamide content. Also, treatment of epidermis with 0.1 N H_2SO_4 on a steam bath for $\frac{1}{2}$ hr., followed by neutralization and extraction with alcohol and ether, failed to reveal the presence of nicotinamide; the latter was easily detected in small amounts of liver treated in the same manner. Finally, hydrolysis of a HPO_3 extract of epidermis with 0.1 N H_2SO_4 failed to yield a detectable amount of this substance. These data, together with the fact that nicotinamide has never been found with the polarograph or paper chromatography in many large samples of this tissue extracted in the usual manner, suggest that this substance is either absent, or present in very minute amounts. The lack or near absence of nicotinamide in epidermis is most unusual, since in pellagra the skin is primarily affected ([11], pp. 246).

Since tumors generally have the same enzymatic systems as normal tissues (6), the alteration or loss of the reducible substance characteristic of epidermis, a very highly specialized tissue, to that of nicotinamide in skin carcinomas might thus be explained. At the present time, our original thesis on a qualitative chemical change in the transformation of epidermis to squamous-cell carcinoma appears still to hold, for reasons listed above, and particularly because nicotinamide has not been found in the former tissue. However, there is no evidence of cleavage of the epidermal compound to smaller components in the carcinomas, as previously postulated (2). This theory was the natural consequence of the fact that epidermis contained only one reducible and UV absorbing substance, while squamous-cell carcinomas contained the latter as nicotinamide and three other UV absorbing materials.

Nicotinamide and creatinine have been extracted from muscle with alcohol and ether mixtures. Creatinine has not been found in the transplanted rhabdomyosarcoma; nor would it be expected to be present in the unorganized structure of this tumor, which has few, if any, characteristics of skeletal muscle (10). In the rhabdomyosarcoma the materials extractable with alcohol and ether are the same as those extracted from the other tumors, with the exception that in about one-half of the former the reducible compound appears to be associated with a substance which absorbs at 350 mμ. The nature of the reducible and UV absorbing materials extractable from liver and hepatoma with alcohol and ether appears to be similar.

SUMMARY

1. Evidence is presented which shows that a polarographically reducible substance characteristic of epidermis is structurally altered or lost in the malignant transformation of this tissue by methylcholanthrene. Nicotinamide and at least three other UV absorbing materials are present in alcohol-ether extracts of squamous-cell carcinomas derived from this tissue. Epidermis shows only one UV absorbing substance which is also reducible, but nicotinamide has not been found in this tissue.

2. Nicotinamide and creatinine were extracted from mouse and horse muscle with an alcohol-ether mixture. In a transplantable rhabdomyosarcoma the latter compound was absent, and in about half of these samples the reducible compound absorbed at 260 and 350 mμ. Whether this yellow material is one or more compounds requires further study. This tumor also had at least three other UV absorbing materials very similar to those of the squamous-cell carcinomas.

3. Mouse liver and a transplantable hepatoma contained nicotinamide and at least three other UV absorbing materials of similar characteristics.

4. A polarographic method for the determination of nicotinamide and possibly other pyridine compounds was briefly discussed.

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The Inactivation *in Vitro* of Transplantable Myeloid and Lymphoid Mouse Leukemic Cells by Antibodies Produced in a Foreign Host Species*

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Natural susceptibility and innate resistance to the development of spontaneous, induced, or transplantable neoplasms are ascribed to differences in genetic constitution (4, 7, 14, 16).[1] The extent to which specific immunogenic processes participate is little understood. The evidence that malignant and normal cells differ in genic composition (5, 9, 10) makes it probable that the antigenic components of these cells likewise should differ. Indeed, it has been shown for mouse leukemia that the injection of leukemic cells results for mice of the same inbred strain in a state of immunity (11) and for animals of heterologous species in the production of antibodies (2, 12, 16). More recently, a variety of immunologic studies has been concerned with malignant cells and their components (1–3). It was with the recognition that immunologic technics make possible more precise information than the application of histopathological and chemical methods that the present investigation was undertaken to demonstrate antigenic differences in the constituents of normal and leukemic cells. It is the purpose of this paper to record the results of experimental studies which show (*a*) the ability of specific preformed antibodies derived from the rabbit to neutralize the leukemogenic effect of leukemic cells on transfer to susceptible inbred mice of known genetic constitution and (*b*) differences in the antigenic components of leukemic and normal cells from the same strain of host.

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[1] For reviews of this complex interrelationship, the reader is referred to references 4, 7, 14, 16.

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MATERIALS AND METHODS

Leukemic cells.—Leukemic cells, line 15, myeloid type, and line I_b, lymphoid type, were employed. Line 15 originated spontaneously in strain F inbred mice in Kirschbaum's laboratory, and line I_b also had its origin spontaneously in the inbred strain C58 in MacDowell's laboratory. Each line has been maintained by serial transmission through many generations. For control purposes, nonleukemic homologous splenic tissues from each of these mouse strains were employed.

Animal hosts.—Line I_b leukemic cells were maintained and tested for viability by injection into C58 inbred mice. These mice were either obtained from Dr. MacDowell or reared in this laboratory. Line 15 leukemic cells were maintained and tested for viability by injection into F_1 hybrid mice which had been derived from the cross of an inbred F male with a female of either the CBA or BALB strain.

Young adult rabbits, mixed sexes, of New Zealand white or pure-bred Dutch strain were employed to produce antileukemic and normal splenic antisera.

Preparation of tissue cell suspensions.—Splenic tissue was utilized for transplantation. A whole spleen was minced with scissors, suspended in 2.3 ml. of 0.85 per cent NaCl and filtered through four layers of Brunswick gauze to remove large clumps of cells. The suspension of cells was counted with the aid of a Levy-Hausser counting chamber to provide, by dilution, a constant number of cells for each injection. The suspension was kept in uniform concentration by continuous mixing. The injection (0.1 ml.) was made intraperitoneally. The inoculum for the myeloid line consisted initially of 100,000 cells. More recently, 10,000 cells were injected per mouse, when it was learned that fewer cells were adequate for a lethal effect. An inoculum consisting of 500 cells of the lymphoid line I_b was employed, when it was learned that this dosage was uniformly lethal for the control animals within

the same incubation period that resulted in death following the injection of 10,000 cells of line 15, myeloid type.

Production of antiserum.—Cellular suspensions were prepared representative of (*a*) lymphatic leukemia splenic tissue from C58 mice inoculated with line I_b; (*b*) normal C58 mouse spleen; (*c*) myeloid leukemia splenic tissue from F_1 hybrid mice inoculated with line 15; and (*d*) normal strain F_1 hybrid splenic tissue. Each rabbit used for production of normal splenic tissue antiserum was given an entire minced spleen suspended in isotonic saline. Since a leukemic spleen is from 3 to 7 times larger than a normal mouse spleen, each rabbit used for production of antileukemic cell serum was given one-third of a minced leukemic spleen. Each of the four inocula was injected intramuscularly into three rabbits. Six 2.0-ml. injections were given to each rabbit at intervals of 72 hours. Five days after the last injection, the twelve rabbits were bled by cardiac puncture. The serum from the three rabbits in each group was pooled, separated by centrifugation at 1,000 r.p.m. for 15 minutes and stored at −10° C.

Technic of neutralization test.—0.2 Ml. of test antiserum was mixed with 0.1 ml. of leukemic cellular suspension. (As noted above, the line 15 leukemic cellular suspension contained either 10,000 cells or 100,000 cells, and the line I_b inoculum consisted of approximately 500 cells.) After each mixture had been kept at room temperature for 1 hour and at 4° C. for a second hour, it was tested in susceptible mice by intraperitoneal inoculation of 0.3 ml. These animals were observed daily for 60 days, to record splenic and lymph node enlargement and the day of death. Mixtures of cells with normal rabbit serum and with 0.85 per cent NaCl, respectively, were prepared for injection for control purposes.

RESULTS

A pilot experiment was carried out as an exploratory measure to learn whether the leukemogenic effect of the test suspension of cells could be neutralized by specific antileukemic cell serum. Both antileukemic serum and normal tissue antiserum rendered mouse myeloid leukemic cells, line 15, nonpathogenic for susceptible test mice, as evidenced by the survival of seventeen out of eighteen test animals. Moreover, an inhibitory effect by normal rabbit serum was manifest, since two of nine recipients survived. These results contrasted with the uniform lethality that resulted for the twenty control mice from the same inoculum.

This observation (that an inhibitory or neutralizing effect upon leukemic cells was common, to a varying extent, in normal rabbit serum, normal splenic tissue antiserum, and leukemic tissue antiserum) made it apparent that normal rabbit serum contained a naturally occurring inhibitor for mouse leukemic cells. In an attempt to eliminate complement and/or a heat-labile inhibitor, each of the test samples of serum was treated by heating for 30 minutes either at 56° C. or at 60° C. The data are presented in Table 1.

It will be observed from the findings in Table 1 that heating for 30 minutes at 56° C. slightly diminished the inhibitory effects of serum samples in all categories. However, treatment of the test serum samples by heat for 30 minutes at 60° C. proved informative. For example, the inhibitory effects of normal rabbit serum were halved and that of normal tissue antiserum reduced to one-third. Contrariwise, the same treatment had only slight effect upon the neutralizing capacity of specific antileukemic rabbit serum. These findings were interpreted to mean (*a*) that a heat-labile inhibitor in normal rabbit serum resulted in the survival of 20 per cent of the recipients despite heating for 30 minutes at 56° C. but in only 12 per cent when heated at 60° C.; (*b*) that normal mouse splenic cells gave rise in rabbits to protective substances which were inactivated by heating to 60° C. for 30 minutes, as evidenced by a survival level of only 21 per cent instead of the 60 per cent survival which had been observed for the recipients that received either unheated serum or serum kept at 56° C. for 30 minutes; (*c*) that myeloid leukemic cells, line 15, were antigenic, to result in neutralizing, protective, heat-stable (60° C.) antibodies that permitted survival of 87 per cent of the recipients.

The results of the experiments summarized on Table 1 were confirmed and extended by the findings that resulted from an essentially similar series of experiments. These experiments differed by the substitution of lymphoid leukemic cells, line I_b, and normal splenic tissue from C58 mice in place

TABLE 1

THE INACTIVATION *in Vitro* OF MOUSE MYELOID LEUKEMIA, LINE 15 BY HEATED RABBIT ANTISERUM

		Treatment of serum samples					
		Unheated		Heated at 56° C. for 30 minutes		Heated at 60° C. for 30 minutes	
		No. mice	Percental survival	No. mice	Percental survival	No. mice	Percental survival
Test Serum Samples	Antileukemic	29/29*	100	12/15	80	21/24	87
	Antinormal tissue	19/29	66	9/15	60	2/24	21
	Normal	8/29	28	3/15	20	3/24	12
Control	0.85 per cent NaCl	0/30	0				

* The denominator signifies the total number of susceptible mice employed as recipients in tests for viability of leukemic cell-antiserum mixture; the numerator, the number of mice that survived without evidence of leukemia.

of the myeloid leukemic cells, line 15, and splenic tissue from F mice.

It can be seen from Table 2 that heating normal mouse spleen antiserum reduced its protective effect, as indicated by a percental survival of 53 for unheated serum, 33 for serum heated at 56° C. for 30 minutes to only thirteen for serum heated for 30 minutes at 60° C. In contrast, lymphoid leukemic tissue antiserum lost, through heating for 30 minutes at 56° C. and at 60° C., only a percental difference of 20. It was assumed that this difference was attributable to a heat-labile inhibitory substance naturally present in normal rabbit serum. The data included in Table 2 for normal rabbit serum do suggest this assumption.

TABLE 2

THE INACTIVATION *in Vitro* OF MOUSE LYMPHOID LEUKEMIA, LINE I_b BY HEATED RABBIT ANTISERUM

		Treatment of serum samples					
		Unheated serum		Heated at 56° C. for 30 minutes		Heated at 60° C. for 30 minutes	
		No. mice	Percental survival	No. mice	Percental survival	No. mice	Percental survival
Test Serum Samples	Antileukemic	14/15*	93	11/15	73	10/15	67
	Antinormal	8/15	53	5/15	33	2/15	13
	Normal	2/15	13	0/15	0	0/15	0
Control	0.85 per cent NaCl	0/20	0				

* The denominator indicates the total number of susceptible mice employed as recipients in tests for viability of leukemic cell-antiserum mixture; the numerator, the number of mice that survived without evidence of leukemia.

DISCUSSION

The studies here described were carried out to demonstrate a difference in the antigenic components of normal and mouse leukemic cells. The *in vitro* neutralization technic commonly employed for the immunological study of viruses was utilized. Rabbits provided the test samples of antiserum. The antigens for the active immunization of rabbits consisted of (*a*) myeloid leukemic tissue representative of line 15, (*b*) normal splenic tissue from strain F mice for control purposes, (*c*) lymphoid leukemic tissue representative of line I_b, and (*d*) normal splenic tissue from C58 mice for control purposes. The present observations suggest strongly that leukemic cells contain an antigenic component(s) distinctive for the mouse leukemic cell. The evidence was found in the repeated demonstration that upon admixture with a homologous leukemic cellular suspension antibodies engendered by leukemic cells protect susceptible mice from an otherwise lethal leukemia. The effect was made apparent by the failure of leukemia to develop in from 80 to 100 per cent of the recipient susceptible mice that were given the test lethal dose of leukemic cells. The antibody for the leukemic cell was heat-stable (60° C. for 30 minutes), in contrast to the relative heat lability (56° C.) for the protective substance(s) that resulted from the antigenic stimulus of normal splenic tissue, or of the protective substances that were found to occur in normal rabbit serum. For example, it was found that heat was destructive for the inhibitory substance naturally present in normal rabbit serum, as evidenced by a loss in protective capacity following heating for 30 minutes at 60° C. It was observed that protection was reduced by heating in one series from 13 per cent to 0 per cent and for another series from 28 per cent to 12 per cent. In contrast to these findings, leukemic cells kept for control purposes for the same periods of time in saline suspension before injection proved uniformly lethal. Cross absorption studies are essential to establish unequivocally the specificity of the leukemic cell antibodies.

The demonstration of specific antibodies for leukemic cells in samples of antileukemic splenic tissue rabbit serum corroborates the results of previous investigators (1–3). Evidence for an antigenic component(s) which differs from the other components of the normal host cell has resulted from the application of a variety of immunological approaches.

The studies of MacDowell, Taylor, and Potter established evidence for the production in susceptible mice of immunity to mouse leukemic cells (11). These investigators immunized mice by utilizing as antigen (*a*) a sublethal dose of leukemic cells or (*b*) normal tissue of a foreign strain. The resistance induced by the injection of splenic or hepatic tissue from mice that had been immunized with leukemic cells was found to be transferable (13). Further studies by these authors (13) revealed differences in the state of immunity that resulted from using leukemic cells for the antigenic stimulus as compared to normal tissue. For example, the specific immunity which had been produced by leukemic cells was transferable to normal animals by the injection of hepatic or splenic tissue from immune animals, but the resistance that resulted from the

administration of normal tissue was not transferable. However, this method for the production of active immunity against transplantable leukemia was not found applicable to spontaneous leukemia, nor would such immunity protect against cells of a spontaneous case (8). These findings by MacDowell and his associates provided a premise for other studies which for the most part were directed toward the elucidation of the mechanism for the inactivation of leukemic cells by antibody or other protective substance.

It is a common observation that the injection of mouse leukemic cells into mice of a foreign strain results in slight evidence of growth and in regression. A second or third transplantation of the same line of leukemic cells does not result in any evidence of growth. Moreover, serum withdrawn from mice rendered immune by this procedure is capable of inactivating leukemic cells *in vitro* so that they are unable to transmit leukemia (6). The injection of leukemic cells, line 15, into a foreign species (15) likewise made available a serum which upon admixture with leukemic cells resulted in their inactivation and in loss of pathogenicity. This experimental study was preliminary to the studies reported in the present paper.

The studies of Dulaney and her associates (1–3) demonstrated quantitative but not qualitative differences in cytoplasmic fractions segregated from the spleen of normal and leukemic mice. These differences were made apparent (1, 3) by utilizing both the complement fixation technic and cytotoxicity (2) for testing antisera which had been prepared by using as antigen the components of normal and of leukemic splenic cells. This demonstration by these two serologic tests of quantitative differences in the test sera is in keeping with the findings of the present study. The test samples of serum in preparation for the serological tests were heated for 30 minutes at 56° C. It seems probable that the differences demonstrated by Dulaney *et al.* might have been better established by heating the serum samples for 30 minutes at 60° C. prior to use.

SUMMARY

Mouse leukemic cells can be differentiated from homologous normal cells by antigenic differences. The neutralization technic demonstrated the ability of rabbit antileukemic tissue antiserum to render a lethal dose of leukemic cells innocuous upon admixture before injection. The specific antibodies were shown to be heat-stable, 30 minutes at 60° C., whereas inhibitory substances demonstrated in normal rabbit serum and in normal tissue antiserum were inactivated at that temperature. The discovery of natural inhibitory substances in rabbit serum makes pertinent the re-examination of the findings of other workers in this field. The lines of leukemic cells employed were myeloid leukemic cells of line 15 from strain F mice and lymphatic leukemic cells, line I_b, in strain C58 mice.

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Nerve Sheath Tumors in an Isolated Goldfish Population*

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INTRODUCTION

During the past 6 years studies have been carried out on the morphology, growth, and possible etiology of nerve sheath tumors observed in goldfish, *Carassius auratus*. The fish were all inhabitants of a large lagoon in the city of Cleveland.[1] The tumors were neurilemomas and neurofibromas; in seven instances they were histologically malignant. The question of the neoplastic character of the melanophores found in several deeply pigmented tumors will be considered.

After an account of the fish and their environment, the morphology of the tumors will be described, followed by data on transplantation and transmission experiments which were carried out. The possible relation of the tumors and certain abnormalities in the fish to constitutional factors, and the similarity of the lesions to those encountered in von Recklinghausen's neurofibromatosis will be presented in the discussion.

MATERIALS AND RESULTS

The Fish and Their Environment

Goldfish visible from the shore of the pond were large, measuring 20–35 cm. in over-all length. An age estimate of 5–8 years was based on studies of the scales made at the Institute of Hydrobiology of the Ohio State University. Fish that bore tumors 1 or more centimeters in diameter were readily spotted as they swam slowly about. They could be lured to within reach of the net by dropping pieces of soda cracker into the water. Occasionally an entire school of fish was caught in a large seine when normal as well as tumor-bearing specimens were needed. Inspection of these fish showed that 8–10 per cent bore one or more tumors. This incidence did not change appreciably during the years of observation. The neoplasms occurred with equal frequency in both sexes. Dead goldfish were seldom found, and none of them bore tumors; it is probable that only rarely did the tumors interfere with the normal life of the fish.

It is regrettable that an ecological survey of the pond has not been made, for it presents a rather unique opportunity to study a restricted yet essentially natural environment of a large number of tumor-bearing animals. The lagoon was stocked 26 years ago, and no goldfish have been added since then. Though the possibility that visitors may have introduced an occasional one must be entertained, no instance of this is known to the caretakers or patrolmen on the grounds.

In addition to the goldfish, blue gill sunfish (*Lepomis incisor*) and large-mouth black bass (*Micropterus salmoides*) are living and breeding in the lagoon. Of those caught in the seine none showed visible evidence of a tumor, nor was there any sign of ocular injury or copepod infestation so common in the goldfish.

The lagoon has a surface area of 3–4 acres; except for three small sandy beaches it is enclosed by low limestone embankments. The water is largely supplied by surface drainage and fountain overflow, supplemented by municipal water during periods of drought. The surrounding lawns are well fertilized, which should aid in maintaining a rich plankton population. There is no evidence of pollution.

The pond is drained to one-third its area each spring, and the marginal zone cleaned for a distance of 20 feet. It requires at least 10 days of constant flow from a city hydrant to refill the lagoon. During the summer months the temperature of the water averages 65° F. (18.3° C.) near shore. In winter the entire surface is frozen, often to a depth of 12 inches, but very few fish die of suffocation.

The Nerve Sheath Tumors

Tumors of the peripheral nerves are among the least common neoplasms found in fishes (30). Of seven papers on the subject, three describe a ganglioneuroma (12, 34, 35) and one a neuroepithelioma (36). Picchi (24) reported a pea-sized tumor that he identified as a schwannoma in the region of a caudal vertebra in a large goldfish. Young and

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[1] Mr. Arnold M. Davis, director of the Garden Center of Greater Cleveland, kindly gave permission for the discriminate use of the fish and supplied data about the lagoon.

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Olafson (42) found multiple lesions in the autonomic nerves of 25 young brook trout. Although the authors describe the pathologic changes as characteristic of neurilemomas, their evidence for the neoplastic nature of the lesions is not entirely convincing.

A comprehensive study of tumors of the nerve sheaths in fishes was made by Lucké (15), who published a detailed description of the gross and microscopic appearance of these neoplasms in 76 specimens of the snapper family (Lutianidae). The affected fish were of three species: *L. griseus*, *L. apodus*, and *L. jocu;* most were caught near the Dry Tortugas in the Gulf of Mexico. The tumors generally occurred along the course of the larger subcutaneous nerves and had the histologic appearance characteristic of neurilemomas seen in man.

Gross Morphology and Distribution

The 144 tumors in the 53 goldfish examined were widely scattered over the surface of the body; however, most were found on the dorso-lateral aspect of the head and trunk and on the caudal fin (Chart 1). Although this is in the area of the lateral line sensory organs and the larger nerve trunks, no tumor attachment to such a nerve could be demonstrated.

The neoplasms ranged in size from 4 mm. to 4.5 cm. in diameter (Figs. 1, 3, 4, 8). The small tumors were flat, orange-yellow in color, and firm in consistency. The larger lesions were hemispherical in shape and broadly sessile; only one was pedunculated (Fig. 5). The surface of the tumors was smooth, sometimes gently lobulated; scales were absent. The neoplasms were usually quite soft, pink in color, and occasionally hemorrhagic (Fig. 2). All tumors were very vascular and bled profusely after injury. A few were pigmented (Fig. 23). Three tumors were cystic; one of these overlay a 2-mm. defect in the skull, but the underlying brain and meninges were intact.

None of the tumors was encapsulated, although

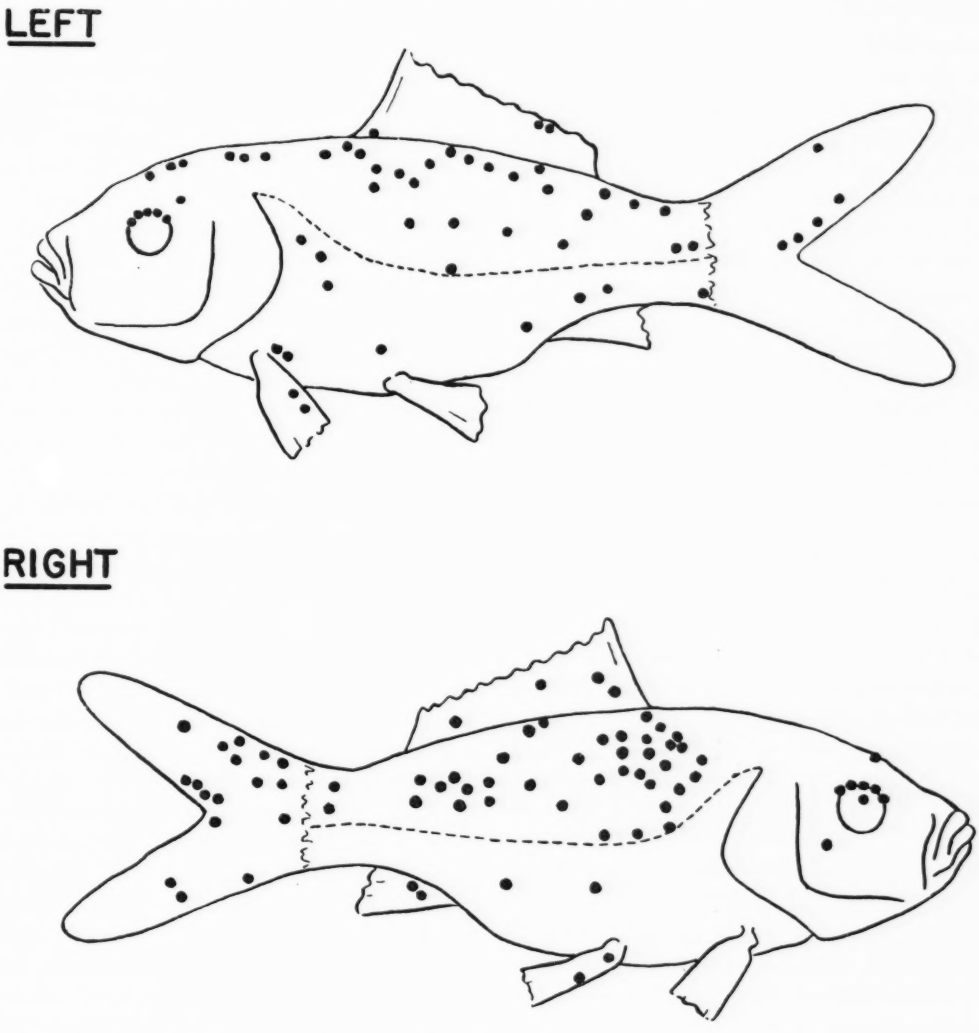


CHART 1.—Distribution of 144 tumors found in 53 goldfish. The tumors occur most frequently on the dorso-lateral aspect of the head and trunk, and on the caudal fin.

most were well circumscribed and did not penetrate the compact layer of the dermis. However, invasion of the underlying musculature (Fig. 31) was observed twice. Metastasis did not occur, but multiple primary tumors were often seen (Figs. 4, 8, 23). Of the 53 fish examined, 31 had two or more tumors; the largest number found on any one fish was eleven.

One goldfish bore no surface lesions, but the right operculum was elevated by a 4 × 4 × 3.5-cm. tumor that arose from the second gill arch on the right (Figs. 15. 16). Another fish had, in addition to three skin tumors, a markedly distended abdomen. After death of the animal, two large contiguous tumors measuring 6.5 × 4.5 × 5 cm. were found to occupy most of the abdominal cavity (Fig. 14). The viscera had been displaced, the ovaries compressed and distorted. The tumors were loosely adherent to the parietal peritoneum; despite careful dissection no other site of origin could be found.

In many tumor-bearing fish careful inspection revealed 1-mm. thick elevations at the tip of isolated scales; the surface was mammilated, orange-yellow in color (Fig. 21). Occasionally, many contiguous scales or a large area of the scaleless head were similarly affected (Fig. 18). The limbus of one or even both eyes was also often the site of diffuse neoplastic thickening (29). Frequently, the lesion encroached upon the cornea (Fig. 9).

Microscopic Morphology

Since the experimental production of neurilemomas by Masson (16) in 1932, much work has been done on the histogenesis of peripheral nerve tumors. These studies, among which those of Stout (31, 33) and Murray and Stout (19, 20) must be reckoned particularly significant, led to the consensus that the chief cellular component of both the neurilemoma and the neurofibroma is the nerve-sheath cell of Schwann rather than the perineural fibroblast. The study of the histology as well as of the growth and development of the goldfish tumors gave results that are compatible with this concept of their histogenesis.

The tumors apparently arose from the small superficial nerves that are very numerous in the corium of the goldfish. Many of the nerves end as naked neurites in the epidermis, others supply end organs known as terminal buds that structurally resemble the taste buds of mammals. These are abundant on the head and trunk of goldfish and other fish of sluggish habit that live in mud (13). The only other specialized end organs in fish are the neuromasts—organs of the lateral line system (1). Histologically, the myelin sheath cells of Schwann are similar to those found in other vertebrates (21). The normal histology of the goldfish skin has been described by Graupner and Fischer (10).

Neurilemoma.—The palisading of nuclei so characteristic of the Antoni type A tissue in the neurilemoma was prominent in 25 per cent of the tumors (Figs. 11, 17) and less apparent in several others (Fig. 7). Although this nuclear arrangement is most often found in nerve-sheath tumors, it occasionally occurs in leiomyomas (33), and in the early stages of this study several tumors were so identified (27). However, further investigation and the accumulation of additional material indicate that these, too, were neurilemomas.

Organoid structures such as the Verocay bodies that resemble tactile corpuscles were not seen. Trichrome stains showed only occasional narrow collagen bundles; silver stains revealed numerous reticulin fibers, but only rarely were neurites encountered. The cells of all type A tissue, whether they showed nuclear palisading or not, were very elongate; cell boundaries were indistinct or absent. The nuclei were uniform in size, long and oval in shape, and contained one to three prominent nucleoli. Mitotic figures were uncommon.

The Antoni type B tissue with its irregularly arranged stellate cells and occasional microcysts (Fig. 6) was less prominent in these tumors than the more compact type A. In several instances there appeared to be a transition between the two types characterized by cells which, though still elongated, had an abundant cytoplasm in which the appearance of vacuoles coupled with intercellular microcysts seemed to foreshadow the loosely arranged meshwork of the fully developed type B tissue (Fig. 10). The blood vessels had thin walls without the collagen sheaths described in some of the human tumors. Multiple thrombi were only encountered in the single pedunculated tumor mentioned above.

Tissue cultures of these tumors were prepared according to a technic similar to that described elsewhere (27). There was often considerable difference in the appearance of the cells from various explants. In some the cells were stellate in shape with an abundant cytoplasm; in other areas of the explant or in other explants of the same tumor the cells approached the extremely long spindle shape (Fig. 36) shown by Murray and Stout (19) to characterize the human Schwann cell. The great pleomorphism of the nerve-sheath cell growing *in vitro* has been emphasized by Weiss (39, 40), who observed their transformation into macrophages.

Neurofibroma.—The thickenings observed at the tip of the scales or diffusely involving the

dorsum of the head have a histological appearance that corresponds well with that currently accepted for the neurofibroma (Figs. 19 and 22). The neoplastic cells, which were cytologically indistinguishable from those of the type A tissue in the neurilemoma, were unlike the latter in their haphazard arrangement. Silver stains showed neurites were present (Fig. 20), a feature characteristic of human cutaneous neurofibromas (18). Neurofibromas and neurilemomas often co-existed on the same fish. When bleached, the histologic appearance of two pigmented tumors resembled that of a neurofibroma.

Pigmented nerve sheath tumors.—Although many tumors had small areas of pigmentation, the four selected for extensive histologic study were gray or black throughout. They occurred on four fish that also bore one or more nonpigmented neurilemomas. Two involved the base of the left pectoral fin, one grew on the caudal fin, and another was found on the dorsum of the trunk (Fig. 23).

In one the pigment was so dense that nuclear and cytoplasmic detail could be made out only at the periphery of the tumor or after bleaching. Growth in tissue culture was poor, but after 13 days in roller tubes small numbers of pigment-bearing cells had migrated out of the explant (Fig. 35). They did not have the branched appearance of melanophores and resembled the pigment-laden macrophages seen in cultures of fish, mouse, and human melanomas by Grand and Cameron (9).

In another neurofibroma the pigmented cells were less numerous and the melanin granules more widely scattered in the melanophores. This permitted a comparison of the nuclei of the pigment cells and of the Schwann cells in the untreated slide (Fig. 24). No distinguishing characteristics were recognizable, and in bleached sections it could no longer be determined which was the pigment cell and which the Schwann cell (Fig. 25).

In a neurilemoma which grossly was diffusely pigmented, histological sections revealed less melanin than was anticipated; typical nuclear palisading was present (Fig. 12). Within the palisades were cells which differed in no way from their companions, except that the cytoplasm contained large numbers of melanin granules (Fig. 13).

The data on the origin of the vertebrate pigment cell (melanophore) has been reviewed by Rawles (25), who concluded that in all classes they were derivatives of the neural crest. More recently, however, Oppenheimer (23) has shown experimentally that in embryos of the fish *Fundulus heteroclitus* pigment cells can arise from cells that normally do not contribute to the teleostean counterpart of the neural crest. Nevertheless, she states that these findings do not rule out the origin of melanophores during normal development from cells corresponding to those of the neural crest in other vertebrates.

Skin pigmentation in the goldfish is subject to considerable change; e.g., it is often increased in areas of inflammation. Goodrich (7) has presented evidence of the existence in the goldfish dermis of a reservoir of melanoblasts (unpigmented precursors of melanophores). From these melanoblasts clusters of melanophores can be derived. Nevertheless, the possibility exists that in the pigmented neurofibromas and neurilemomas of the goldfish the melanophore is derived from the same stem cell as is the Schwann cell and that it is an integral part of the tumor. This intimate relation of the two cell types recalls Masson's concept of pigmented moles (17) which he believes are composed of a downward proliferation of intraepidermal melanoblasts and upward migration of Schwann cells from the dermal nerves.

The pigment-producing ability of Schwann cells has been described in certain ocular melanomas by Reese (26), who labeled the tumors "neurogenic melanomas." A similar histologic picture was observed by the writer in a malignant melanoma that arose in the body wall of a python (30).

In the light of Weiss's studies on the *in vitro* transformation of the Schwann cell into a macrophage (39, 40), the possibility should be considered that the melanin-bearing macrophages described above (Fig. 35) are melanophores similarly altered rather than macrophages that have phagocytized the pigment of disintegrating melanophores in the explant.

Malignant neurilemoma.—The histology of the nerve sheath tumors thus far considered has been that of benign neoplasms characterized by the absence of any profound cellular abnormality. However, in seven fish there were rapidly growing tumors that showed histological evidence of malignancy. All were large, three were solitary, neurofibromas accompanied the other four. One of the tumors apparently arose in the right eye which it had destroyed, another grew on the dorsum of the head, a third occupied a large area of the operculum behind the right eye (Fig. 8). The remaining four were located on the dorsolateral aspect of the trunk (Fig. 40).

The histologic picture presented by these tumors was quite variable. It differed essentially from that of the benign tumors in the more abundant cytoplasm of the cells and the frequent appearance of giant forms with bizarre nuclei and prominent nucleoli (Fig. 26). Normal and abnormal mitotic figures were often seen (Figs. 27 and

28). In several instances the chromosomes, which normally are very small with a diploid number of 90, were large, resembling the diplochromosomes described by Biesele (2) in an ovarian tumor of the goldfish (Fig. 29).

The neoplastic cells were arranged as interlacing bundles in which there was occasionally a suggestion of nuclear palisading (Fig. 32). Areas similar to the Antoni type B tissue of the benign neurilemoma were encountered. Silver stains failed to reveal the presence of neurites, and reticulin stains did not show the encircling reticulin fibers deemed characteristic of fibrosarcoma (32). The histologic appearance of these tumors in the goldfish closely resembles that of malignant neurilemomas recently reported in man (8, 38).

Invasion of the underlying musculature was only observed twice (Fig. 31); no metastases were found. The only instance of the successful transplantation of a tumor in this entire series was an autotransplant in one of these seven cases (Figs. 37–39).

Several of the malignant tumors grew well in tissue culture. Although they rarely displayed the very elongate form of human Schwann cells *in vitro*, many had the interesting "flask" shape (Fig. 34) and monocytoid character (Fig. 33) observed by Weiss in Schwann cells from the nerves of adult rats. (Compare with Weiss's Figures 5 and 6, Plate I [40].) However, whether the monocytoid cells represent morphological variants of the Schwann cells or macrophages present in the tumor explants could not be determined.

Rate and Manner of Growth

The benign tumors grew slowly; e.g., a neurilemoma on the caudal fin (Fig. 1) showed no demonstrable increase in size over a period of 5 months and no growth of explants in tissue culture. A pigmented neurilemoma at the base of a pectoral fin manifested no change during the $7\frac{1}{2}$ months the fish was under observation. A pigmented neurofibroma in an identical location on a second goldfish showed regrowth after portions were excised for tissue culture but only a slight enlargement of the entire tumor. Very poor growth was obtained *in vitro*. During the 14 months that this animal was in the laboratory a partly pigmented 4-mm. tumor on the operculum regressed until only a flat pigment spot remained. A small tumor on the trunk showed no change. In another case a pedunculated neurilemoma (Fig. 5) was amputated 2 days after capture of the fish. Tissue cultures of the tumor were contaminated. After 57 days there was evidence of recurrence, but the mass measured only $4 \times 2 \times 6$ mm. This was removed and pieces cultured *in vitro*, where growth was poor. During the remaining 8 months of life there was no recurrence of the tumor at the primary site. The best *in vitro* growth of a benign tumor was obtained with a neurilemoma that arose from the second gill arch (Figs. 16 and 36).

The seven tumors that showed histological evidence of malignancy all grew rapidly during the 32–203 days they were under observation (Figs. 2 and 3). In one instance the tumor appeared to outstrip its blood supply, for the bulk of it became necrotic and sloughed off. Viable tumor continued to grow about the margins of the resultant ulcer. A tumor that was observed for 203 days displayed alternating periods of slow and rapid growth. Biopsies taken from four of these seven tumors yielded good growth in tissue culture.

Transplantation Experiments

Numerous auto-, homoio-, and heterologous transplants of the benign as well as of the histologically malignant tumors were made. Only a single one, an autotransplant to the anterior chamber of the eye, showed evidence of progressive growth. This failure of the transplants to grow, coupled with the absence of metastases, would indicate that none of the goldfish tumors had reached the stage of autonomous growth (11).

The technic used in transplanting tissue to the anterior chamber of fish eyes is similar to that employed with other animals. One difficulty should be noted; *viz.*, the anterior chamber fluid of the goldfish is very viscid and escapes through the wound for several minutes after the sclero-corneal incision has been made, often carrying the transplant with it. This was overcome by introducing the tumor with a Bashford needle rather than with forceps and by placing the transplant at the most inferior portion of the chamber. During the procedure, which only lasted 3–4 minutes, each fish was wrapped in a damp cloth with only the head protruding. No ill effects from the resultant partial asphyxia were observed.

Autotransplants in the anterior chamber.—A bit of the primary neoplasm was transferred to one eye of the tumor-bearing fish in five instances, Nos. 1, 5, 6, 8, and 28. All but the last were histologically malignant neurilemomas; No. 28 was a benign neurilemoma arising from a gill arch. Growth occurred only in goldfish No. 1; the implants in Nos. 6 and 28 were resorbed within 4 weeks. No. 8 lived only 6 days after inoculation, and, in the case of No. 5, failure of growth may have been due to ocular trauma incurred during the operation.

Growth of the autotransplant in fish No. 1 was

not apparent until 3 months after inoculation; during this period the bit of tumor was visible in the lower half of the anterior chamber where it rested upon the iris. In the 3 weeks before the animal's death, when the primary tumor was growing rapidly, the transplant began to increase in size and grew into the pupillary space (Fig. 37). Histological sections showed that the transplant had become attached to the iris which supplied it with blood vessels (Fig. 38). In their structure and arrangement the cells of the transplant resembled those of the primary tumor (Fig. 39).

A small incision was made at the base of a scale just lateral to the dorsal fin; through this a Bashford needle was inserted and the tumor transplants placed in the subcutaneous tissue. Material from two malignant neurilemomas was used. That of goldfish No. 5 was inoculated into seventeen fish obtained from dealers and that of goldfish No. 29 was transferred to eight goldfish from the Cleveland pond. No growth of the transplants took place; all were completely resorbed.

Heterologous transplants in the anterior chamber.—Pieces of a neurilemoma (G. F. 28) were transplanted to the anterior chamber of the left eye in 24 frogs. A moderately severe inflammation characterized by haziness of the cornea set in after 24–48 hours. A week later the cornea partly cleared and the tumor tissue was visible in the anterior chamber. It had become white and opaque and subsequently was completely resorbed. Homoiotransplants of this tumor had also failed to grow (Table 1), although growth in tissue culture was excellent (Fig. 36).

TABLE 1

HOMOIOTRANSPLANTS IN THE ANTERIOR CHAMBER

Identity of donor	Type of tumor	No. of Cleve.* fish	No. of Cleve.* fish with tumor	No. of dealer fish	Remarks	Growth of transplant
G.F. 6	Malignant neurilemoma			12	In right eye	0
				12	In left eye 4 wk. after inoc. of tumor G.F. 6 in right eye	0
				12	Fish inoc. subcut. with tumor G.F. 10 one month before	0
G.F. 21	Malignant neurilemoma		4	35	Inflammation in eyes of tumor-bearing fish	0
G.F. 29	Malignant neurilemoma	8		24		0
G.F. 44	Malignant neurilemoma	5	3	7		0
G.F. 28	Benign neurilemoma	11	3	20		0
				8	Fish inoc. 1 wk. before in other eye with tumor G.F. 27	0
		16			Fish inoc. 12 days before in other eye with tumor G.F. 27	0
		10			Transplants were tissue cultures	0
G.F. 27	Neurofibroma	16		8		0
Total		66	10	138		0

* "Cleve." in all tables refers to the Cleveland lagoon.

Homoiotransplants in the anterior chamber.—Tissue from several rapidly growing tumors was transplanted to the eyes of goldfish obtained from the Cleveland lagoon; these may have been genetically related to the donors. In a few instances the recipients bore spontaneous nerve-sheath tumors, a situation which in similar studies with rodents has increased the number of takes (11). Additional host fish were purchased from dealers to provide animals in which any genetic relation between donor and recipient would be extremely unlikely (Table 1).

In several instances one eye was inoculated with the same or a different tumor 1–4 weeks before transplants were placed in the other eye. No effect was noted upon the rate of growth or regression that could be compared to the XYZ phenomenon of Casey (4). In one experiment explants from tissue cultures were used, but these too regressed.

The inflammatory response to the transplant was very slight; clouding of the cornea seldom occurred, and infection was rare. The majority of the animals were observed for 3 months or more; in none did the transplants grow, nor did tumors develop elsewhere in the inoculated fish.

Homoiotransplants in the subcutaneous tissue.—

TRANSMISSION EXPERIMENTS

In view of the high incidence of these tumors among goldfish in the Cleveland lagoon, it appeared that they might be induced by a transmissible agent. To test this possibility the following procedures were carried out.

Subcutaneous inoculation of tumor suspensions.—A virus or other carcinogenic agent might not be liberated from the intact cells of the tissue used in transplantation experiments. Pieces of the tumor were therefore ground with sand in isotonic saline (0.8 per cent). After the sand and other coarse particles had settled out, the supernatant fluid with its suspended tissue debris was injected intramuscularly near the dorsal fin of healthy goldfish.

Tissue from the malignant neurilemomas of goldfish Nos. 1, 10, and 27 was prepared as outlined and injected respectively into ten, twelve, and eight dealer's goldfish. In addition material from fish No. 27 was similarly injected into sixteen normal goldfish obtained from the Cleveland lagoon.

Although over half of the 46 inoculated animals survived more than 5 months, none developed tumors at the site of injection or elsewhere.

Association of normal with tumor-bearing fish.—Transmission of a carcinogenic virus or other agent by accidental inoculation is unlikely in natural surroundings. To determine the existence of other modes of transmission, tumor-bearing fish were placed in large tanks with healthy goldfish obtained from the Cleveland pond and from dealers (Table 2).

TABLE 2

TUMOR-BEARING FISH IN TANKS WITH NORMAL FISH

No. of tumor fish in tank	Size of tank (gal.)	No. of Cleve. fish in tank	No. of dealer's fish in tank
5	140	12	15
1	20		8
5	140	12	15
5	140	11	20

The aqueous environment of fish in the same tank lends itself to the transmission of disease agents. The urine and feces excreted into the water are taken up by the fish in the process of forcing water over its gills during respiration. That some of the water is also swallowed was demonstrated in these goldfish by visualization of the intestine in roentgenograms made after exposure of the fish to water containing thorium dioxide, with the method of Frank and Allee (6). In addition, the excreta, other body discharges, and exfoliated cells suspended in the water come in contact with the entire skin surface of the fish.

Although many normal fish, both from the Cleveland pond and from dealers, were kept in tanks with tumor-bearing fish for over 8 months, none of the healthy fish developed a tumor. It is possible that a carcinogenic agent present in the water of the lagoon is not transferred with the tumor-bearing fish. This can perhaps be determined by placing normal goldfish, restrained in wide-mesh screen cages, in the Cleveland lagoon where they may be examined at intervals for the appearance of tumors.

Copepod infestation and neoplasia.—Many tumor-bearing as well as healthy goldfish from the Cleveland pond were parasitized by copepods (Fig. 40) of the species *Lernaea carassii* described by Tidd (37). Other species of these minute parasitic crustaceans, colloquially known as "anchor worms," are often observed on fresh water or marine fishes where they may produce prominent inflammatory tumefactions (22).

The larvae of *L. carassii* pass through five nauplius stages in which they are free-swimming, then follow six copepodid stages during which the larvae move about over the surface of the fish, occasionally leaving one and transferring to another. At the end of the sixth copepodid stage the animals molt and develop into the adult form. The young adult burrows through the epidermis into the corium of the fish, after which six hooks rapidly develop about the copepod's rostrum and serve as anchors. Meanwhile the abdominal segments elongate and dangle freely in the surrounding water. In the female two egg sacs, in which the early stages of development occur, grow from the caudal segment (Fig. 42).

The adult crustacean produces a considerable degree of local inflammation with hyperemia and small hemorrhages in the corium. At 25° C. the entire life cycle requires about 2 weeks, but the adult parasite remains imbedded in the host for several weeks. If infestation occurred in the fall it may still be found on the fish the following spring.

This life cycle of the parasite, coupled with the frequent presence of the adults on tumor-bearing fish and occasional appearance in the tumors themselves (Fig. 41), suggested that the parasite might be important in the genesis of the tumor. The copepod could transmit a virus or a chemical carcinogen, or by producing a chronic inflammatory lesion provide a stimulus that in a congenitally susceptible strain would lead to tumor formation.

To test these possibilities mature female copepods were removed from tumor-bearing fish, the egg masses collected, and the larvae reared to the first copepodid stage, when they were brought in contact with the fish to be parasitized.[2] Although some fish became heavily infested, an attempt was made to keep the number down to 10–15 adults per fish to facilitate mapping their location and noting changes at those sites during the following months.

In the first experiment 24 dealer's goldfish were heavily infested with copepods (Fig. 43). When the infestation was at its height and there was danger that the fish might die, they were treated with potassium permanganate. The infestation promptly subsided, and after a week the parasites had disappeared. Although half the fish survived more

[2] Grateful acknowledgment is made for valuable advice given by Prof. Wilbur M. Tidd on the technique employed.

than 6 months after infestation, none developed tumors.

Further studies were then carried out using both tumor-bearing and nontumor-bearing fish from the Cleveland pond as well as dealer's goldfish (Table 3). The fish with tumors were used in

TABLE 3

COPEPOD INFESTATIONS

Animals parasitized	No. of animals	Water temp. (° C.)	Length of survival (mo.)	No. of survivors
Dealer's fish	50	25	6+	23
Dealer's fish	50	15–25	6+	25
Cleve. fish	40	15–25	0– 1	2
			1– 2	5
			2– 3	3
			3– 4	5
			4– 5	6
			5– 6	3
			6– 7	1
			7– 8	3
			8–10	12
Cleve. fish with tumors	15	15–25	0– 1	2
			1– 2	3
			2– 3	3
			3– 4	3
			4– 5	0
			5– 6	3
			6– 7	1

the event that a virus might be present in the tissues of the fish that would be activated by the wound, as is true of some plant tumor viruses (3). Because these experiments were carried out in the fall of the year, it was possible to cool the water sufficiently to check the infestation without resort to potassium permanganate.

Although approximately half of the animals survived over 6 months after infestation, no tumors were observed to develop at the site of injury produced by the copepod. Histologically, an acute inflammatory response in the host tissue enveloped the parasite. After several weeks the body of the parasite sloughed out and the wound healed, leaving no trace of previous injury.

CHRONIC IRRITATION AND TUMOR GENESIS

All the fish used in the copepod experiments and listed in Table 3 were identified by a numbered metal clip attached to the operculum (Fig. 4). This served as a chronic irritant to the delicate gill filaments immediately beneath the tag, to the body surface behind it, and to the operculum in which the tag was fastened. The gill tissues responded with chronic inflammatory changes followed by repair in which hyperplasia of the pavement epithelium was a prominent feature. Granulation tissue formed about the clip in the operculum, but nothing that suggested neoplastic change could be recognized. No evidence for activation of a virus or other carcinogenic agent was obtained.

GENETIC FACTORS IN TUMOR GENESIS

The possibility that genetic factors may play a role in the development of these peripheral nerve tumors must be seriously entertained. A consideration of this problem will be presented in the discussion.

A pair of tumor-bearing fish were bred; of the offspring about 150 are living and are now 2 years old. No tumors have been seen on any of the fish, and, except for the occasional occurrence of a shortened or "pug" head, no somatic abnormalities were encountered. Since most of the tumor-bearing fish were over 5 years old when captured, it is possible that tumors will yet appear in the young goldfish.

DISCUSSION

Neurofibromatosis of von Recklinghausen in man is characterized by the occurrence of multiple cutaneous and visceral neurofibromas and neurilemomas with focal areas of pigmentation in the skin. Associated with these classic lesions there may be a variety of neuroectodermal tumors and developmental anomalies (14).

In some measure the features of human neurofibromatosis are reproduced in these goldfish. In the fish as in man the common neurogenic tumors are cutaneous neurofibromas and neurilemomas; visceral nerve sheath tumors are occasionally found. Malignant neurilemomas are encountered in about 10–15 per cent of the cases in both man and the goldfish. The melanoblasts found in some human neurofibromas are often abundant in the tumors of the goldfish. The possible relation of these pigmented neurilemomas of the fish to the pigmented moles of man (quite frequent in von Recklinghausen's disease) has already been noted. The diffuse involvement of the corium found in some patients with neurofibromatosis produces a great thickening of the skin called elephantiasis neuromatosis. A histologically similar though grossly much less pronounced lesion has been seen on the head and anterior trunk of several goldfish (Figs. 18 and 19).

In addition to the nerve-sheath tumors two interesting abnormalities were observed in these goldfish; *viz.*, polycystic kidneys (28) and buphthalmos (29). Of eleven fish with polycystic kidneys, three bore neurofibromas or neurilemomas. The cysts varied in size and in one of the three tumor-bearing fish filled and greatly distended the abdominal cavity (Fig. 23). In this instance the fish had five cutaneous neurofibromas, of which two were pigmented. Renal malformations have also been described in patients with von Recklinghausen's disease (41).

Neurofibromas at the sclero-corneal junction are of frequent occurrence in these fish (29). The tumors were nearly always unilateral and in ten of twelve cases were associated with nerve-sheath tumors elsewhere on the body. Other ocular abnormalities resembling keratoconus and buphthalmos in man were observed in 20–30 per cent of the goldfish from the Cleveland lagoon. These congenital abnormalities and ocular neurofibromas have also been seen in human cases of neurofibromatosis (5).

In conclusion, the high incidence of nerve sheath tumors, some intimately associated with pigment cells, as well as the occurrence of developmental abnormalities in these isolated and probably inbred goldfish, resembles von Recklinghausen's neurofibromatosis in man. A genetic background, recognized in the human patient with this syndrome, may also be present in the goldfish.

SUMMARY

Approximately 8–10 per cent of the goldfish in an urban lagoon exhibit nerve-sheath tumors. Of 53 tumor-bearing fish, 31 had two or more tumors. These were classified as neurilemomas and neurofibromas; seven were histologically malignant. Melanophores were present in several of the neoplasms; the question of the neural crest origin of both the pigment cells and the Schwann cells was considered. All the tumors were subcutaneous with two exceptions: in one fish two large neurilemomas occupied the abdominal cavity, in another a neurilemoma arose from the second right gill arch.

Growth in tissue culture was often abundant. Acquisition of the morphological characteristics of a macrophage by nerve sheath cells growing *in vitro* as reported by Weiss is also suggested in cultures of the neoplastic Schwann cells of the goldfish.

The auto-, homoio-, and heterologous transplants of this tumor failed to grow, with the exception of a single autotransplant to the anterior chamber of the eye. Attempts to determine the presence of a carcinogenic agent by transmission experiments, including infestation with a parasitic copepod, were likewise negative.

Congenital anomalies including polycystic kidneys, keratoconus, and buphthalmos occurred in some of the tumor-bearing fish as well as in otherwise normal goldfish from the same pond. The frequency of congenital lesions served to emphasize the fact that the fish were an isolated population probably subjected to considerable inbreeding and therefore had some genetic homogeneity. Although 2-year-old offspring of a pair of tumor-bearing fish are still free of tumors, a possible genetic background for both the tumors and the anomalies should be considered and a comparison drawn with von Recklinghausen's neurofibromatosis in man.

ACKNOWLEDGMENTS

I am indebted to Mrs. Leonard Paul for technical assistance in the preparation of histologic sections and tissue cultures, and to Joe D. Humphrey for the photographs. The Ohio Division of Wildlife, Section of Fish Management, loaned valuable equipment for use in these studies.

FIGS. 1–7: Representative tumors.

FIG. 1.—A neurilemoma on the caudal fin; the tumor, which measured 3×2×2 cm., slowly increased in size over a period of 123 days. (Goldfish 7.)

FIG. 2.—A hemorrhagic and histologically malignant neurilemoma that arose on the trunk immediately behind the operculum. (Goldfish 5.)

FIG. 3.—Photograph of fish shown in Figure 2 taken 25 days later; rapid increase in size of the tumor is apparent. Tissue removed at this time grew well *in vitro*, but attempts at auto- and homoiotransplantation were unsuccessful. (Goldfish 5.)

FIG. 4.—A neurilemoma on the snout and another on the dorsum of the tail anterior to the caudal fin; histologically both tumors showed nuclear palisading. The fish was observed for 110 days; during that interval there was only slight increase in size of the tumors. Growth in tissue culture was poor. (Goldfish 30.)

FIG. 5.—A pedunculated neurilemoma. During the 10 months following amputation of the tumor at the base of the pedicle there was only slight local recurrence. Growth in tissue culture was poor. (Goldfish 38.)

FIG. 6.—Loose reticulated area from preceding tumor resembling Antoni type B tissue of human neurilemoma. Hematoxylin and eosin (H. and E.) stain. Mag. ×200. (Goldfish 38.)

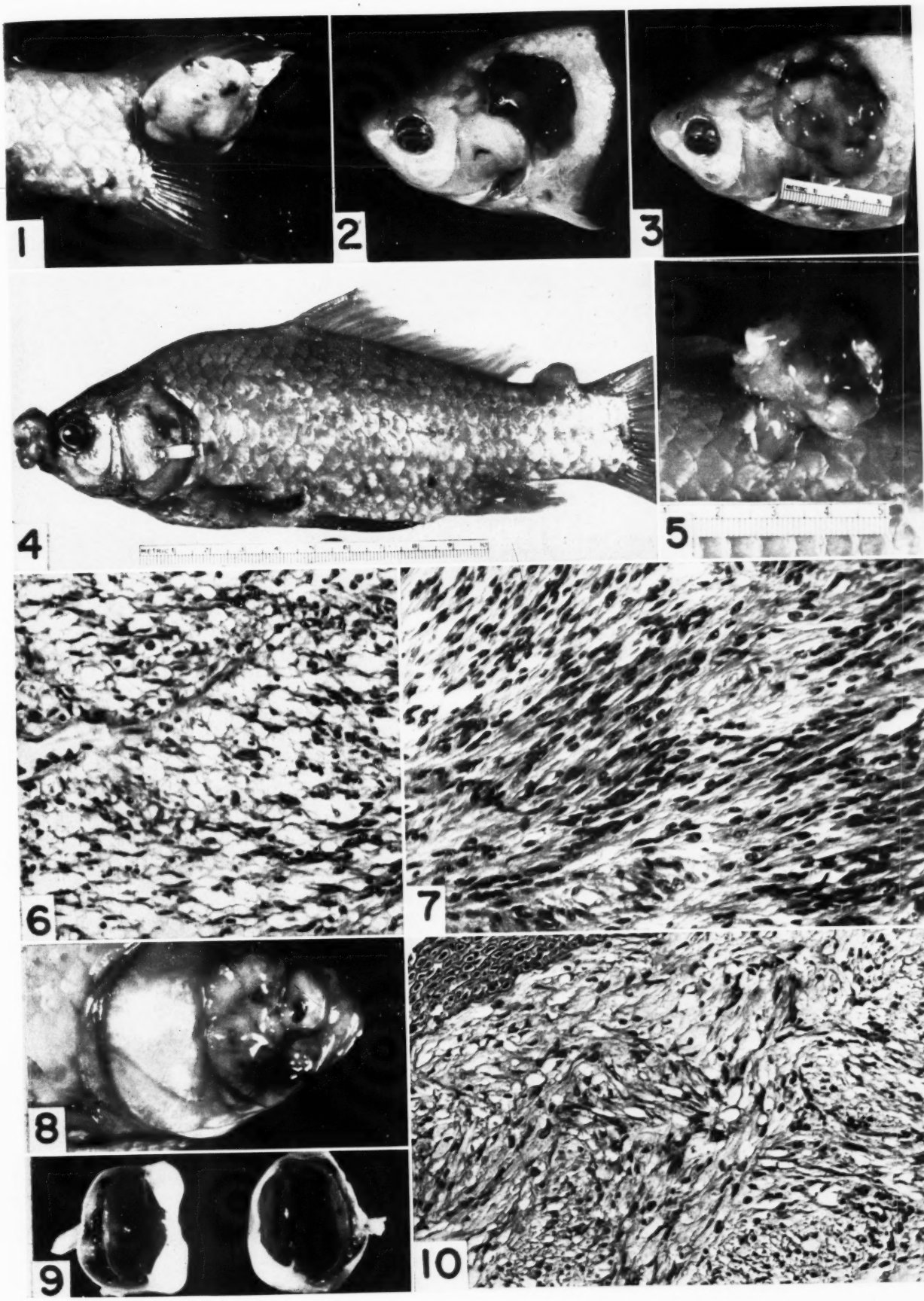
FIG. 7.—A different area from the same tumor, showing fasciculate arrangement of cells and a suggestion of nuclear palisading. This may be compared to Antoni type A tissue of human neurilemoma. H. and E. stain. Mag. ×250. (Goldfish 38.)

FIGS. 8–10: Ocular neurilemomas.

FIG. 8.—A large, crescentric, very soft, hemorrhagic tumor involving most of the right cheek directly behind the eye. The tumor measured 3×2×1.5 cm. The cornea of the eye is thickened at its periphery by tumor tissue. Attempted homoiotransplantation of the cheek tumor was unsuccessful although it had the histological character of a malignant neurilemoma. (Goldfish 44.)

FIG. 9.—Saggital section through the eyes of the goldfish shown in Figure 8. The cornea of each eye is greatly thickened by a neoplastic growth. (Goldfish 44.)

FIG. 10.—Tumor replacing normal connective tissue of cornea shown in preceding figure. The corneal epithelium is in the upper left corner. The cytoplasm of the neoplastic cells is abundant, often vacuolated. Small intercellular vacuoles (microcysts ?) suggest a transition between tissue of Antoni type A and type B in the neurilemoma. H. and E. stain. Mag. ×200. (Goldfish 44.)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FIGS. 11–13: Neurilemomas with prominent nuclear palisading.

FIG. 11.—Section of a 6×8×4 mm. neurilemoma that grew at the base of the caudal fin. Palisading of nuclei is very prominent. Van Gieson stain. Mag. ×105. (Goldfish 32.)

FIG. 12.—A pigmented tumor measuring 10×8×7 mm. grew at the base of the left pectoral fin. Section shows an area of nuclear palisading; branched pigment cells (melanophores) are absent. Bodian stain. Mag. ×135. (Goldfish 36.)

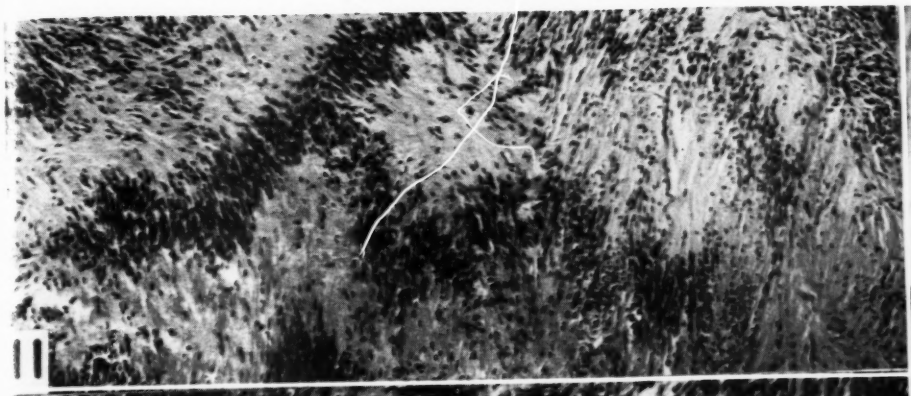
FIG. 13.—Portion of an area of nuclear palisading shown in the preceding figure. Several of the sheath cells contain large numbers of melanin granules in their cytoplasm. See also Figures 23–25. Bodian stain. Mag. ×335. (Goldfish 36.)

FIGS. 14–17: Visceral neurilemomas.

FIG. 14.—Two large neurilemomas occupy most of the abdominal cavity. The intestine is displaced forward by the tumors and the swim bladder "B" lies above them. (Goldfish 49.)

FIGS. 15 and 16.—Lateral and ventral views of a tumor that arose from the second gill arch and elevated the operculum. The tumor grew well in tissue culture, but homoiotransplants were resorbed. (Goldfish 28.)

FIG. 17.—Section of the neurilemoma shown in Figures 15 and 16 is composed predominantly of the loosely arranged stellate cells of Antoni type B tissue. See Figure 36 for manner of growth in tissue culture. Mag. ×165. (Goldfish 28.)



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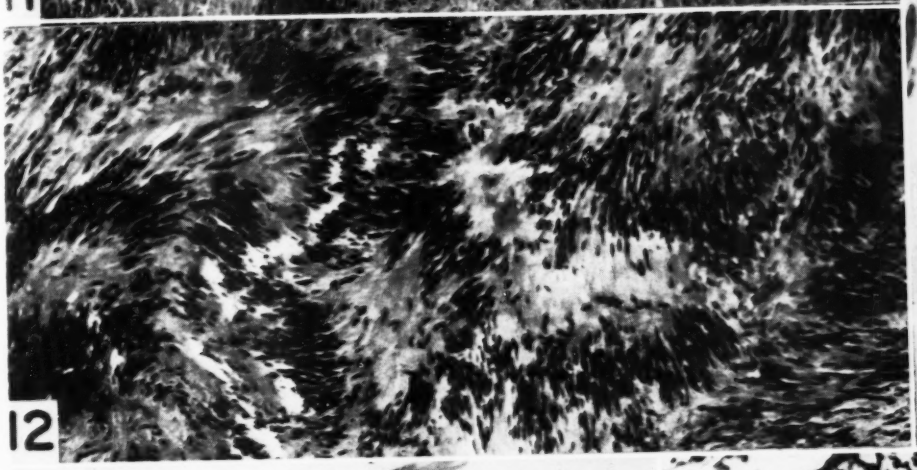

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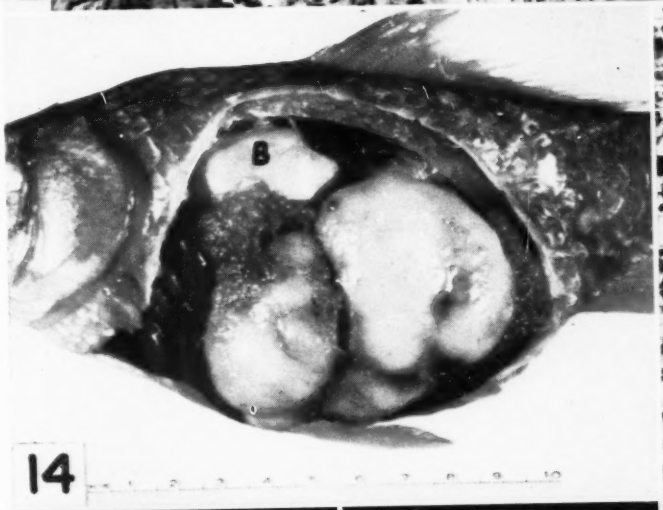

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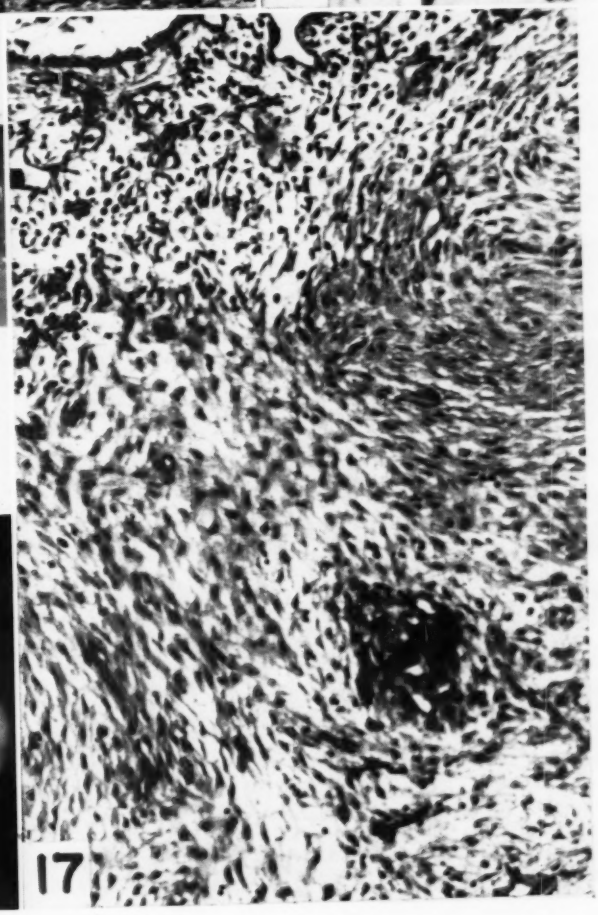

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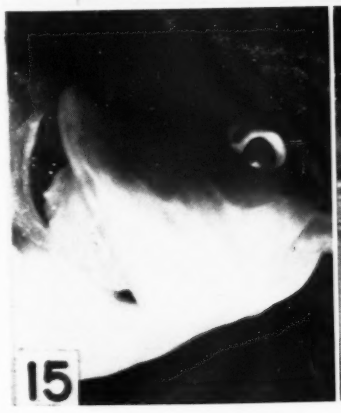

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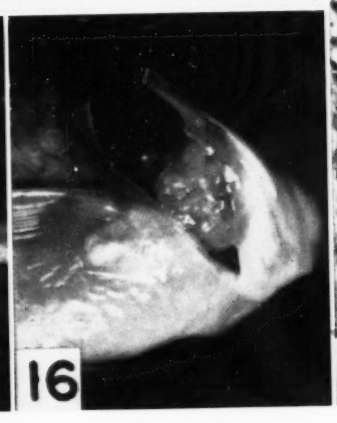

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FIGS. 18–22: Dermal neurofibromatosis.

FIG. 18.—Goldfish showing nodular skin on the dorsum of the head. Clouding of the cornea is due to neurofibromatous infiltration. (Goldfish 98.)

FIG. 19.—Section through nodular area of skin shown in preceding figure. The connective tissue of the corium has been replaced by irregularly arranged bundles of Schwann cells separated by greatly elongated pegs of epithelium. This may be compared to the diffuse neurofibromatosis identified in man as elephantiasis neuromatosa. H. and E. stain. Mag. ×25. (Goldfish 98.)

FIG. 20.—Neurite in tumor area shown in Figure 19. Bodian stain. Mag. ×300. (Goldfish 98.)

FIG. 21.—Single scale showing a mammilated dermal thickening at its tip. Mag. ×5. (Goldfish Kid. 7.)

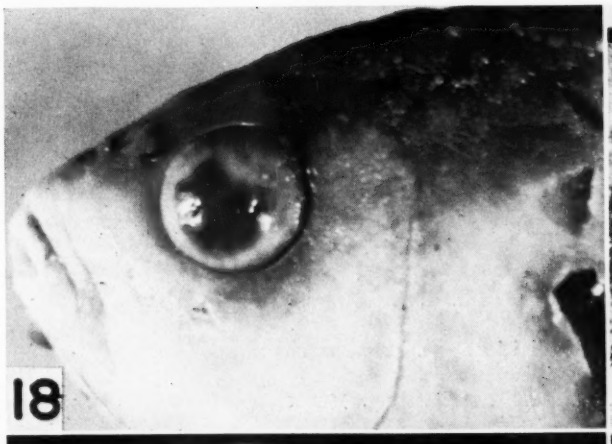
FIG. 22.—Section taken through thickening at tip of scale shows a diffuse proliferation of Schwann cells. The overlying epidermis is thin and composed chiefly of large clear clavate cells except where epithelial pegs extend downward. The lesion probably represents an early stage of that shown in Fig. 19. H. and E. stain. Mag. ×75. (Goldfish Kid. 7.)

FIGS. 23–25: Pigmented neurofibromas.

FIG. 23.—The abdomen is greatly distended by large bilateral polycystic kidneys (28). On the dorsum of the head, near the midline, is a flat nonpigmented neurilemoma. On the trunk are three pigmented neurofibromas, the largest of which lies just to the left of the midline immediately behind the head. (Goldfish 21.)

FIG. 24.—Section of a pigmented tumor from the base of the left pectoral fin of goldfish No. 39. Much of the tissue pattern is obscured by large branching melanophores. H. and E. stain. Mag. ×200.

FIG. 25.—Same as Figure 24 but bleached before staining with hematoxylin and eosin. The cellular pattern is that of a neurofibroma. Mag. ×200. (Goldfish 39.)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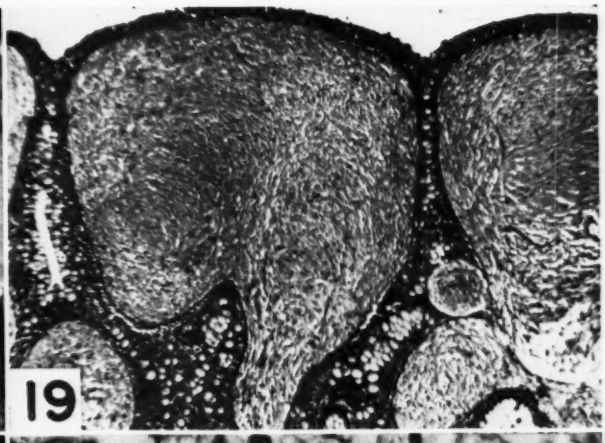
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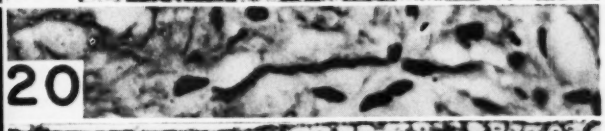
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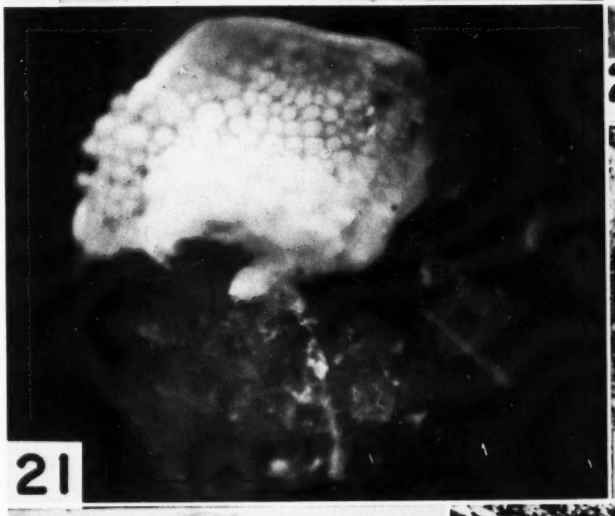
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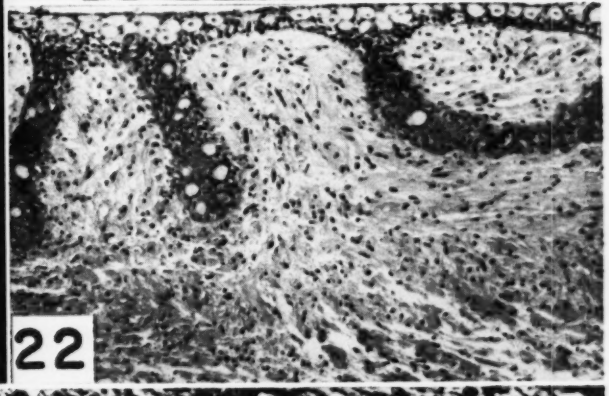
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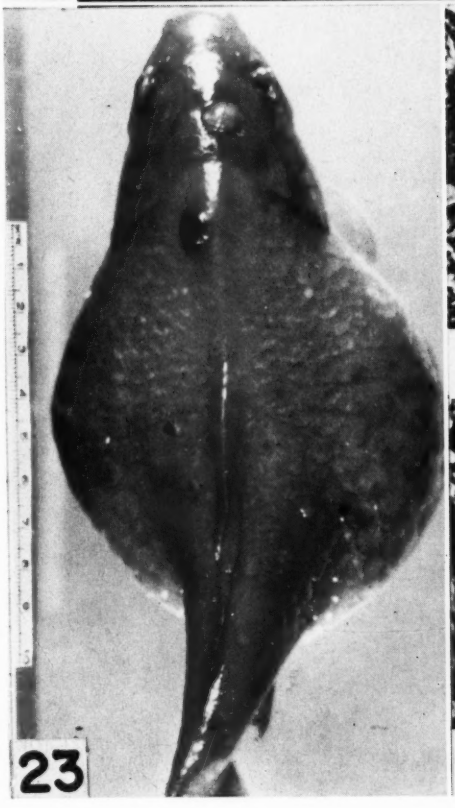
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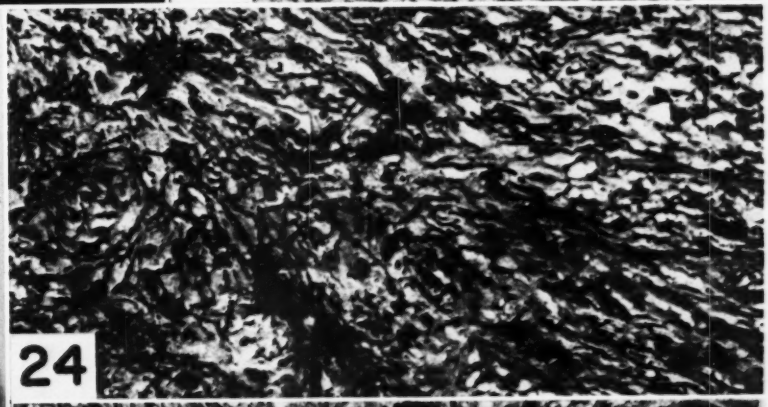
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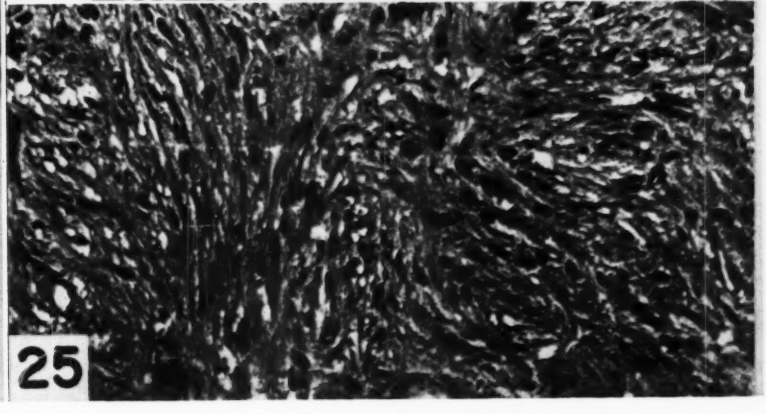
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FIGS. 26–32: Malignant neurilemoma.

FIG. 26.—Binucleate tumor giant cell. H. and E. stain. Mag. ×800. (Goldfish 21.)

FIG. 27.—Tripolar mitosis in large tumor cell. H. and E. stain. Mag. ×800. (Goldfish 1.)

FIG. 28.—Abnormal mitosis in tumor cell. H. and E. stain. Mag. ×800. (Goldfish 1.)

FIG. 29.—Tumor cell in mitotic division. The chromosomes are larger than those normally seen in the goldfish and may represent diplochromosomes. H. and E. stain. Mag. ×800. (Goldfish 5.)

FIG. 30.—Section of a soft hemorrhagic tumor that occupied the dorsum of the tail directly behind the caudal fin and measured 3×4×2.5 cm. The cells are elongate, aligned in bundles; the nuclei are variable in size and shape. H. and E. stain. Mag. ×210. (Goldfish 21.)

FIG. 31.—Invasion of trunk muscles by tumor cells. The tumor grew on the right dorso-lateral surface of the body, in front of the dorsal fin. A central ulcer 2 cm. in diameter was surrounded by an elevated margin of yellow-white tumor tissue. H. and E. stain. Mag. ×80. (Goldfish 37.)

FIG. 32.—Malignant neurilemoma showing evidence of nuclear palisading. The tumor arose from the limbus of the right eye which it destroyed in a period of 5 months. Transplants to the eyes of other goldfish failed to grow. H. and E. stain. Mag. ×300. (Goldfish 29.)

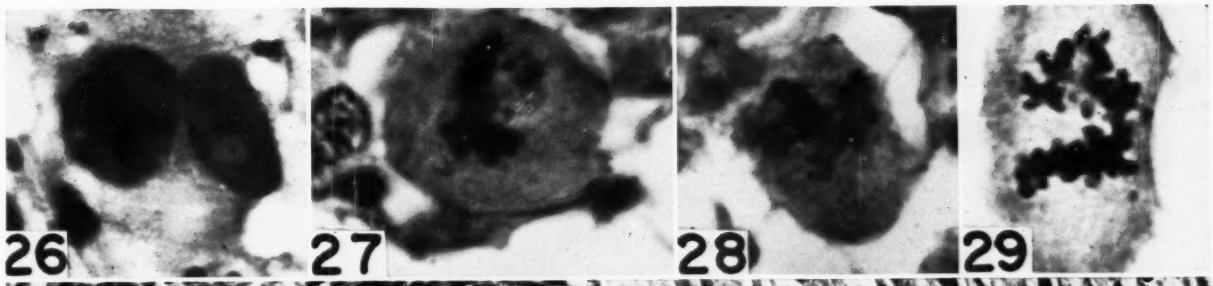
FIGS. 33–36: Growth of tumors *in vitro*.

FIG. 33.—Cells wandering out of tumor explant after 4 days *in vitro*. Whether these cells represent macrophages or monocytoid Schwann cells cannot be determined. Compare with Weiss, 1944, Figure 6. H. and E. stain. Mag. ×450. Culture on coverslip. (Goldfish 1.)

FIG. 34.—Growth of neoplastic Schwann cells after 6 days *in vitro*. Many are elongated and some are flask-shaped with the nucleus at one end. Compare with Weiss, 1949, Figure 5. H. and E. stain. Mag. ×300. Culture on coverslip. (Goldfish 1.)

FIG. 35.—Cells bearing melanin granules leaving explant after 13 days *in vitro*. These cells probably represent macrophages that have phagocytized melanin liberated from degenerating melanophores in the explant. However, see text for discussion and Fig. 23 for appearance of tumor. H. and E. stain. Mag. ×450. Culture in roller tube. (Goldfish 31.)

FIG. 36.—Growth, after 7 days *in vitro*, of tissue from tumor that arose on a gill arch (Figs. 15–17). The cells have assumed an elongate shape approaching that characteristic of human Schwann cells growing *in vitro*. H. and E. stain. Mag. ×400. Culture in roller tube. (Goldfish 28.)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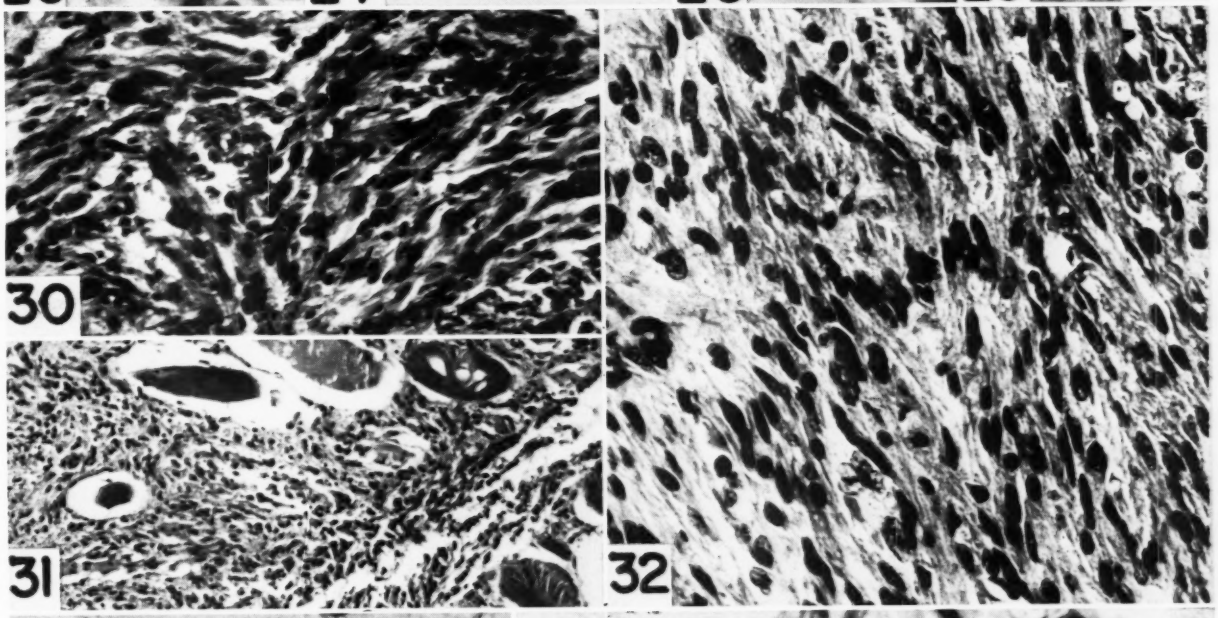
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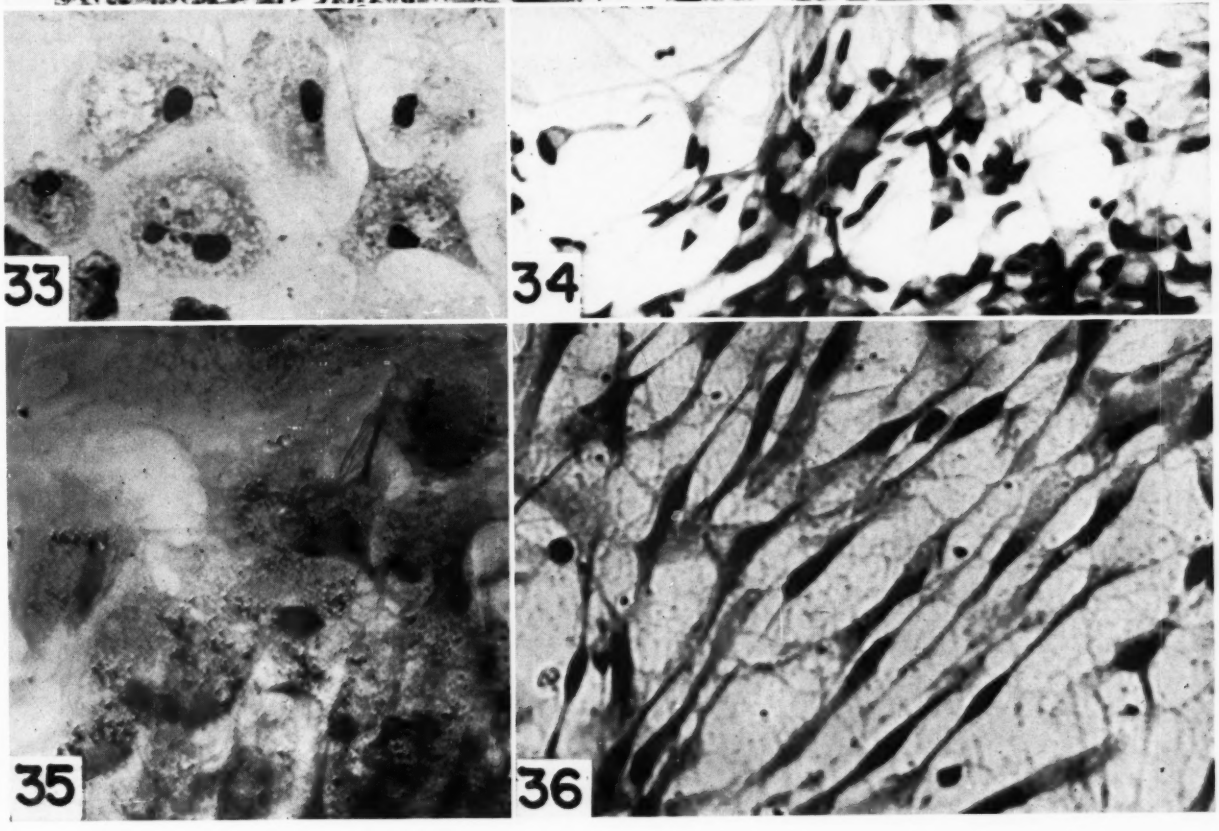
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FIGS. 37–39: Autotransplant of neurilemoma.

FIG. 37.—Tumor transplant in anterior chamber of eye after 3 weeks rapid growth that followed 3 months of quiescence. The primary tumor was a histologically malignant neurilemoma that grew on the head of this fish. Mag. ×4. (Goldfish 1.)

FIG. 38.—Histological section of transplant showing its attachment to the iris. H. and E. stain. Mag. ×10. (Goldfish 1.)

FIG. 39.—Higher magnification of section shown in the preceding figure. The tissue was clearly viable at the time of fixation and closely resembles the histological appearance of the primary tumor. H. and E. stain. Mag. ×310. (Goldfish 1.)

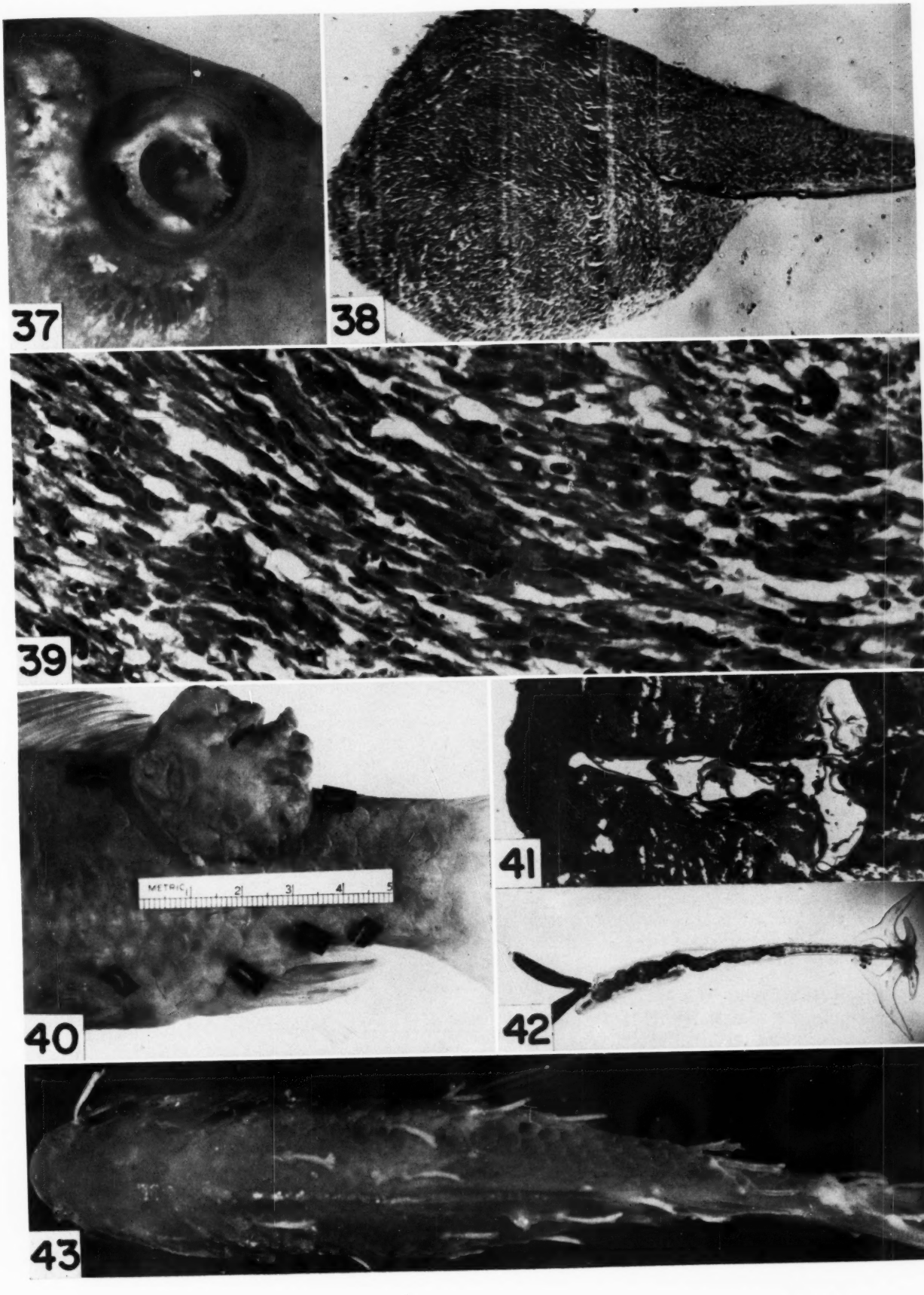
FIGS. 40–43: Copepod infestation and neoplasia.

FIG. 40.—Large neurilemoma on tail of goldfish. Scattered over the trunk and tail are numerous adult copepods; a bit of black paper was placed under several to make them more distinct. (Goldfish 8.)

FIG. 41.—Section of tumor shown in previous figure. The plane of the section passes lengthwise through an adult copepod embedded in the tumor. H. and E. stain. Mag. ×10. (Goldfish 8.)

FIG. 42.—Adult female copepod; the two egg cases are seen at the caudal end; on the right are the large T-shaped hooks about the rostrum. Mag. ×8.

FIG. 43.—Dealer's goldfish showing heavy experimental infestation with copepods. Natural size.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Neoplastic Diseases in Infants and Children*

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The predominant malignant neoplasms of infants and children arise from tissues of mesenchymal origin, in contrast to the characteristic epithelial malignant tumors of adults. A sound explanation for this apparent influence of age on tumor types would be a significant contribution to oncology. It has been suggested that age alone has little effect on the development of sarcomas and that the incidence of sarcomas varies only slightly from age 15 to age 75, if percentage corrections are made on the proportionate number of persons living in various age periods (21). However, age is definitely important in the development of carcinomas. They are rare in infancy and childhood, begin to be prominent aberrant growths in late youth and early adult years, and reach their peak of frequency in the middle decades of life. Their increasing incidence with age supports the concepts of carcinogenesis in the human that were so ably presented by Cramer (4). If one accepts the ramifications of his theories and recognizes the importance of the time element in carcinogenesis, it is not difficult to understand why carcinomas are not prominent neoplasms in the first decade of life.

Explanations for the origin of human carcinomas have not proved adequate for the origin of the sarcomas. Even though the belief that childhood tumors arise from embryonal rests continues to receive support (1, 12), it is not inconceivable that an influence on mesenchyme in intra-uterine life is equally important, thus explaining the greater numbers of malignant tumors reported in the first 5 years of life than in the subsequent two 5-year periods. On the other hand, if the incidence of sarcoma does not vary significantly in later age periods as has been stated, it is difficult to understand how the effects of an adverse intra-uterine milieu could be delayed until late adult life.

Our limited knowledge of the methods of origin of malignant neoplasms of infants and children may be due in part to their rarity. Farber (10) has stated that the 301 histologically verified malignant tumors, excluding the leukemias, studied during a recent 10-year period at The Children's Hospital in Boston constituted only 0.6 per cent of the total admissions. Certainly, such tumors do not form a significant component of the illnesses in the average pediatrician's practice or of a pediatric or surgical service in a general hospital. Although Duzan (9) as early as 1876 analyzed 182 cases that had been recorded in the literature from 1832 to 1875, subsequent reports were sporadic (5, 16, 22, 30), and, even as late as 1947, material was collected from only nine institutions (6). It included a "combined" series of 1,770 cases of childhood cancer from seven institutions, a group of 116 fatal malignant tumors from the Chicago Memorial Hospital, and 103 fatal tumors from the University of Chicago (29). Together with discussions of 506 malignant tumors by Dargeon in 1948 (7), of 467 by Fleming and Pearce (11) in 1949, of 144 by Anderson and Martin (2) and 768 benign and 175 malignant tumors by Andersen (1), both in 1951, they constitute the recent major surveys of neoplasms in infants and children.

Stimulated by knowledge of their rarity and of the benefit that may be derived from further surveys of this character as additive source materials on the subject, we are herein reporting the benign and malignant tumors seen at the James Whitcomb Riley Memorial Hospital, the children's hospital at the Indiana University Medical Center.

The disease categories admitted to any medical institution will in a certain sense be selected and influenced by such factors as its geographic location, racial and other characteristics of the patient constituency it serves, and the scientific interests of its professional personnel. Andersen has illustrated this selection by showing how the administration of her institution makes her case material deficient in tumors of the eye, central nervous system, and bone. The Riley Hospital has also selected its patients, but in a unique manner. First, it is a state institution and accepts only a minimal number of patients from outside Indiana. Second, it is administered for the care of the indigent patient, and nearly all the children admitted to it have been from families who were not economically favored. Within these limitations, it has admitted children 15 years of age and under.

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The institution was opened in November, 1924, and from then to June 30, 1951, there were 85,895 admissions to it. There were 1,409 patients with tumors, a number that is probably a significant numerical and medical sample of all children's tumors in this state in the 27-year period.

The age and sex distribution of the major pathological groups are indicated in Table 1. The

TABLE 1

SEX AND AGE DISTRIBUTION

Character of tumor and age periods	Males	Females	Totals
Benign:			
Under 1 yr.	108	210	318
1– 4 yrs.	97	95	192
5– 9 "	61	62	123
10–15 "	94	111	205
Totals	360	478	838
Malignant:			
Under 1 yr.	9	9	18
1– 4 yrs.	51	35	86
5– 9 "	27	20	47
10–15 "	36	35	71
Totals	123	99	222
Leukemia:			
Under 1 yr.	8	12	20
1– 4 yrs.	47	26	73
5– 9 "	20	10	30
10–15 "	15	10	25
Totals	90	58	148
Brain and spinal cord:			
Under 1 yr.	8	2	10
1– 4 yrs.	40	26	66
5– 9 "	35	30	65
10–15 "	32	28	60
Totals	115	86	201
GRAND TOTALS:	688	721	1,409

benign neoplasms and the sex distribution of the total series were weighted heavily by the hemangiomas, 388 of which were recorded. Two hundred and forty-six of them occurred in infants under 1 year of age, 174 in females and 72 in males. Although females dominated in the total hemangioma category in a proportion of over 2 to 1, this distribution does not necessarily represent, in our opinion, a truly greater incidence of hemangiomas in females, but perhaps a recognition by parents of a potentially greater economic liability in later life of a disfigured female child. The weighting from the female hemangioma group was in contrast to the sex incidence in the other groups where in general it was noted that males predominated.

The anatomic classification of all categories of neoplasms is indicated in Table 2. In addition to skin, the hemangiomas contributed a large proportion of the benign neoplasms of somatic soft tissues and the oral cavity, as well as eight of the benign neoplasms of the genito-urinary system because of involvement of the external genitalia. Dermoid cysts, fibromas, and naevi were prominent examples of other benign skin tumors. The lymphangiomas, including the cystic hygromas, contributed 49 of the benign tumors of somatic soft tissue. Others were lipomas, fibromas, and neuromas. Benign tumors of bone were osteochondromas, chondromas, cysts, osteomas, and giant-cell tumors. Dermoid cysts accounted for sixteen of the eighteen benign tumors in the eye and orbit.

TABLE 2

ANATOMICAL CLASSIFICATION

Anatomical area	Benign	Malignant	Total
Skin	451	5	456
Somatic soft tissue	142	42	184
Hematopoietic system		148	148
Bone	101	44	145
Oral cavity and pharynx	54	8	62
Genito-urinary system	16	38	54
Eye and orbit	18	26	44
Larynx and lung	36	2	38
Lymphoid tissue		29	29
Endocrine glands	10	12	22
Gastro-intestinal system	5	10	15
Nasal cavity		6	6
Breast	5		5
Brain			190
Spinal cord			11
Totals	838	370	1,409

Thirty-five of the 36 in the larynx and lung group were papillomas of the larynx. All ten in the endocrine system were adenomas—eight in the thyroid and one each in the parathyroid and adrenal. Three of the gastro-intestinal tract group were carcinoids of the appendix, and two were polyps of the large intestine. All the breast tumors were fibroadenomas.

The malignant neoplasms are classified anatomically in Table 2 and pathologically in Table 3. The lymphoid tissue group includes only the cases where lymph nodes seemed to be involved primarily and does not include the lymphosarcomas that seemed to arise elsewhere, such as in the intestine. All the lymphomas and the leukemias constituted 192, or 51.6 per cent, of the 370 malignant neoplasms, a larger proportion than has been reported in some of the other series. On the other hand, the malignant bone group is smaller in proportion than Dargeon's combined series (6), accounting for 11.6 per cent as contrasted to his 20.6 per cent. Twenty-one of the malignant genito-urinary tumors were Wilm's tumors of the kidney that have been reported by Garrett and Mertz (13). Eight of the remainder were sarcomas of the bladder, kidney, prostate, and uterus; three were

carcinomas of the testicle; and two each were malignant teratomas of the ovary and testicle and unclassified carcinomas of the kidney. In addition to the retinoblastomas, there were two malignant melanomas of the eye, one neuro-epithelioma, one spongioblastoma, and one reticulum-cell sarcoma

TABLE 3

PATHOLOGICAL CLASSIFICATION

Malignant Neoplasms

Leukemia		148
Sarcoma		87
Unclassified	31	
Osteogenic	23	
Fibrosarcoma	20	
Rhabdomyosarcoma	5	
Round cell	3	
Fibromyxosarcoma	2	
Leiomyosarcoma	1	
Kaposi's sarcoma	1	
Neurogenic	1	
Lymphoma		44
Lymphosarcoma	28	
Reticulum-cell sarcoma	8	
Hodgkin's disease	8	
Retinoblastoma		21
Wilm's tumor		21
Carcinoma		12
Neuroblastoma		12
Ewing's tumor		10
Malignant teratoma		8
Malignant melanoma		5
Neuroepithelioma (retina)		1
Spongioblastoma (optic nerve)		1
Total		370

of the orbit. Sixteen lymphosarcomas, eight Hodgkin's disease, and five reticulum-cell sarcomas comprised the group of lymphoid tissue tumors that we considered arose in lymph nodes. All the malignant neoplasms in the endocrine glands were in the adrenal gland—ten neuroblastomas, one carcinoma, and one unclassified sarcoma. Among the malignant gastro-intestinal tumors there were two carcinomas of the colon; a carcinoma and an unclassified tumor of the liver, five lymphosarcomas and an unclassified sarcoma. Two of the nasal cavity malignant neoplasms were carcinomas, and four were unclassified sarcomas.

The pathological classification of the neoplasms exclusive of the benign tumors and of those arising in the brain and spinal cord clearly indicates the importance of the leukemias. The tumors whose tissue of origin could not be identified, but which clearly were sarcomatous, are listed as unclassified sarcomas. The relative infrequency of the carcinomas is emphasized in Table 3, and the organs from which they originated are indicated in Table 4. It is of some interest that we have seen no carcinomas of the thyroid, although Horn and Ravdin (17) report only four cases in patients 15 years of age and under.

The pathological classifications of the neoplasms in the brain and spinal cord are indicated in Table 5. Tissue was available on two of the 56 unclassified tumors of the brain, but it could not be classified. It was not available in the remaining 54, but 27 of them died with disease. The remaining 27 could not be traced. Tissue was also studied pathologically on the three unclassified tumors of the cord, but in none could a specific diagnosis be made. All three patients died. The incidence in the brain is somewhat different from that described by Bailey *et al.* (3), who found that the astrocytoma was the most common tumor in this age group, followed in order by the medulloblastoma, spongioblastoma, and ependymoblastoma.

TABLE 4

PHYSICAL DISTRIBUTION OF CARCINOMAS

Location	No. cases
Testicle	3
Kidney	2
Nose and nasopharynx	2
Transverse colon	1
Sigmoid colon	1
Liver	1
Skin of face	1
Adrenal	1

TABLE 5

PATHOLOGICAL CLASSIFICATION OF BRAIN AND SPINAL CORD TUMORS

Pathological type	Brain	Spinal cord
Medulloblastoma	42	
Astrocytoma	35	1
Spongioblastoma	29	2
Ependymoma	7	1
Pinealoma	7	
Craniopharyngioma	6	
Hemangioblastoma	4	1
Papilloma of choroid plexus	3	
Neuroma		2
Meningioma	1	1
Unclassified	56	3
Totals	190	11

An analysis of the incidence of the four types of neoplasms in relation to the total tumor admissions and classified in 3-year periods is presented in Chart 1. The changing importance of the leukemias in this 27-year period is of interest and is statistically significant. In the 1927–1930 period, for example, there were five leukemias admitted, whereas from 1948 to 1951 there were 41. In an associated study of the mortality records of the Indiana State Department of Health by Mr. Vern Robinson for the years 1924–1950, inclusive, it was found that there were 474 reported deaths from leukemia and 636 from other malignant tumors. The increasing frequency with which fatalities

from these diseases are recorded in his analysis is illustrated by the fact that in the 3-year period, 1924–1926, there were 63 reported deaths from malignant tumors and 23 from leukemia, whereas in the period 1948–1950 there were 113 and 111 deaths, respectively. The increase in the reported leukemia deaths was particularly striking in the age group of 4 years and under where it rose from ten to 71, whereas in the age period 5–14 years it rose only from 13 to 40. The population of the state increased 27.5 per cent in the same period of time, although information on the change in age groups is not available.

DISCUSSION

The discovery of carcinogenic agents and their application to human cancer has illuminated the method of origin of many human carcinomas. The fact that the carcinoma is a rare tumor in infants and children supports the theory of carcinogenesis in so far as the time factor is concerned. Furthermore, the frequency of all types of neoplasms—benign, malignant, leukemia, and brain and spinal cord (Table 1) in the first 5-year period of life, as compared to the two subsequent 5-year periods—suggests either an adverse environment in intra-uterine life or shortly thereafter or an aberration of embryology.

Although the studies of Gregg (15) that associated congenital cataracts and rubella and the experiments of Duraiswami (8) with insulin showed that intra-uterine environment could be responsible for congenital deformities of the new-born, the origin of congenital neoplasms in the infant human has not been correlated with a similar abnormal environment. With the exception of tumors of retinal origin, Wells (31) was able to accept without reservation only 66 of the congenital neoplasms reported in the literature. As a possible explanation for other neoplasms in infants, he suggested that there may be some relationship between the type of metabolism represented by the high glycolytic activity of such tissues as brain and renal medulla and the occurrence of such tumors as the neuroblastoma and nephroblastoma.

Nevertheless, the origin of the majority of the malignant neoplasms in children is unexplained, even though sensitivity of embryonic tissue to carcinogenic agents has been demonstrated. Attention was called to this characteristic by Greene (14) in 1945 in a report of a study of the carcinogenic action of methylcholanthrene on homologous transplants of embryonic lung, stomach, intestine, skin, muscle, and cartilage. It appeared significant to him that such embryonic tissues underwent carcinogenic changes within 35 days, whereas 90–200 or more days were required for similar changes in adult tissue.

The extensive investigations of Smith and Rous (23, 25, 26, 27) of the same problem have shown that embryonic lung, epidermis, and stomach are not only sensitive to the carcinogenic activity of methylcholanthrene when transplanted to an adult host, but also to benzpyrene and dibenzanthracene. Moreover, embryonic prostate and thyroid seem to share the sensitivity.

Of even greater interest with respect to our present report are the experiments with urethan that indicate it will act through the placenta as a carcinogenic agent to the embryo. Larsen (20) found that when pregnant strain A mothers were treated with urethan, their offspring, at 6 months of age, had developed lung tumors in significantly greater numbers than the controls; and that the

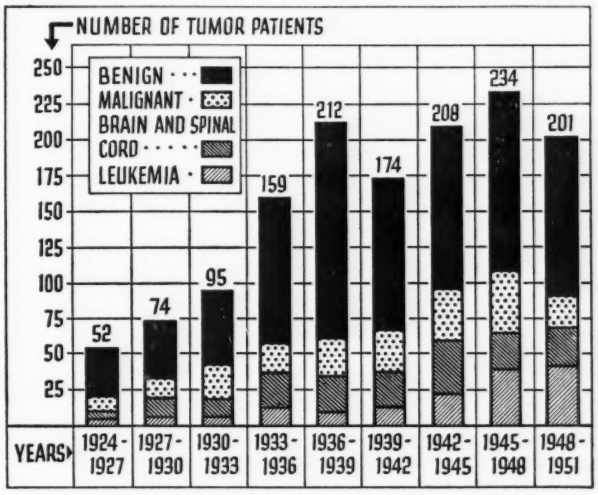

CHART 1.—Tumor admissions grouped in 3-year periods

incidence of tumors was greatest in the group born within 24 hours of treatment. Smith and Rous (28), working on the same problem with strain C mice, several times noted pulmonary adenomas in mice sacrificed at 3 days of age. At 10 days the adenomas had attained considerable size. Furthermore, Klein (19) has indicated that mice related to the strain A animals that were delivered by caesarean section and fostered by untreated mothers had essentially the same frequency of pulmonary tumors as those that were nursed by treated mothers. Such data assume even greater importance when considered with the preliminary evidence presented by Shay *et al.* (24) that malignant lymphomas developed in young rats nursed by mothers fed methylcholanthrene by stomach tube.

It thus seems clear that several types of embryonic tissue have a high degree of sensitivity to

carcinogenic agents and that some carcinogenic agents can be transmitted to the embryo through its mother.

Perhaps these experimental carcinogens, or others, have a similar effect on the human embryo if properly activated through the mother. The acute leukemias are an example of a disease that may have such an origin and offer interesting possibilities for further epidemiological and clinical experimental studies. The fact that radiant energy and benzol poisoning may cause leukemia in the adult (18) suggests similar influences in the unborn infant.

The increasing rate of admissions of patients with leukemia to the Riley Hospital and of deaths from leukemia in the state should not necessarily be construed as indicating an increasing incidence of the disease in children, but may be owing only to better recognition of it. Nevertheless, leukemia appears to be a disease of ever greater importance, and, since methods for its control are totally unsatisfactory, it would seem prudent to exert a more intensive study of its epidemiology and of its possible origin from carcinogenic stimulation in the prenatal period.

SUMMARY

1. A series of 1,409 patients with benign and malignant tumors seen at the James Whitcomb Riley Hospital from November 1, 1924, to July 1, 1951, is presented.

2. Anatomic and pathologic features of the group are tabulated.

3. Theoretical explanations for the greater incidence of neoplasms in the first 5 years of life than in the subsequent 5-year periods are discussed.

4. The increasing admissions of the leukemias and the increasing reported death rate from them in the state of Indiana have been discussed and possible explanations for them presented.

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The Influence of Dietary Casein Level on Tumor Induction with 2-Acetylaminofluorene*

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INTRODUCTION

The present experiments were conducted to determine the carcinogenicity of 2-acetylaminofluorene when it was fed in partially purified diets that varied in casein level between 9 and 60 per cent. Studies involving this broad range of dietary protein level have not been reported previously with this carcinogen.

Harris (7) concluded that variations in dietary protein level had no appreciable effect on the production of liver tumors with 2-acetylaminofluorene in rats. Purified diets containing 13 and 20 per cent of casein were employed. Morris and associates (10) found that rats developed more tumors when purified diets containing 18–24 per cent of casein were used than when the diet contained 12 per cent of casein.

In these studies, it was noted that dietary protein levels in the range used by the above investigators had no influence on tumor induction by 2-acetylaminofluorene. However, it was observed that diets containing 40 or 60 per cent of casein had a definite protective effect against the induction of mammary tumors and possibly other types of tumors.

PROCEDURE AND RESULTS

Weanling female rats of the Alabama Experiment Station (AES) strain, 40–60 gm. in body weight and 20–22 days old, were used. They were kept in individual screen-bottomed cages. Fresh feed and water were supplied daily, ad libitum. A record of feed consumption was kept during the first 16 weeks of the experiment. The animals were weighed and examined for visible tumors at weekly intervals.

The basal diet had the following percentage composition: alcohol-extracted casein, 9.0; degerminated corn grits, 20.0; sucrose, 50.7; salts,[1] 4.0; lard, 15; L-cystine, 0.3; and cod liver oil, 1.0. Fifty ml. of an aqeous solution containing the following vitamins (mg.) was mixed into each kilogram of the dry ingredients: thiamin, 2; riboflavin, 4; pyridoxine, 6; calcium pantothenate, 10; niacin, 20; *i*-inositol, 200; and choline chloride, 2,000. Alpha-tocopherol and α-tocopheryl acetate were mixed into the cod liver oil to furnish 25 mg of each/kg of diet. All diets contained 300 mg/kg of 2-acetylaminofluorene, which was added to the fat-free dry ingredients in acetone solution and the solvent removed with an air current.

Increases in the casein component of the diet were at the expense of equal weights of the sucrose component. The other dietary changes that were tried are given in Table 1.

The basal diet is similar to diet C-5 used in earlier studies (2–5), except that the supplement of pyridoxine was increased from 2 mg to 6 mg/kg of diet to eliminate the possibility of the occurrence of a deficiency of this vitamin on the high casein diets. Varying the pyridoxine supplement between 2 and 10 mg/kg of diet was demonstrated in preliminary trials to be without influence on the carcinogenicity of 2-acetylaminofluorene.

The animals were continued on experiment until they died, unless indicated otherwise. At termination, all tumors and grossly abnormal tissues were carried through routine procedures for histologic study; a complete description of the autopsy material will appear in another publication.

The present results confirm the earlier observations (2–5), in that a high incidence of mammary tumors was obtained when the 9 per cent casein diet was fed (Table 1, groups 1 and 11). When this diet was fed, liver and ear duct tumors appeared

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[1] W. D. Salmon, J. Nutrition, **33**:155, 1947.

with about the same frequency as reported earlier.

Increasing the dietary protein to 12, 20, or 27 per cent had no influence on the final incidence of mammary, ear duct, or liver tumors (groups 2, 3, and 6). However, when the dietary casein was increased to 40 or 60 per cent, there was a marked reduction in the incidence of mammary tumors (groups 4 and 5) when the food consumption was about equal to that on the lower protein diets (groups 1 and 11). When the 60 per cent casein diet (group 5) was used, three out of nine animals remained tumor-free for 40 weeks, whereas on the low protein diets all animals had tumors of one or more types, usually by the 25th week, and the average survival was only 27 weeks.

In contrast with the marked reduction in mammary tumors in group 5 is the high incidence of mammary tumors in group 9, which was also fed the high casein diet but which consumed larger amounts of feed and gained weight more rapidly.

To test the relation of mammary tumor induction to body weight gain and feed consumption level, the study represented by groups 11, 12, and 13 was conducted. Animals in groups 11 and 13 were allowed to consume their diets ad libitum. The animals in group 12 were paired with those in group 11, and the feed offered to those on the 60 per cent casein diet (group 12) was limited to the amount consumed by those on the 9 per cent casein diet (group 11). With equalized caloric intakes (viz., equalized feed intakes), the 60 per cent protein diet again resulted in a marked protection against mammary tumor induction by 2-acetylaminofluorene (group 12). With ad libitum feed consumption of the 60 per cent casein diet, a moderately high incidence of mammary tumors again occurred (group 13).

Irrespective of the level of feed consumption and the growth rate, the 60 per cent casein diets had a real and beneficial effect on survival. Animals receiving this diet survived 40 weeks or longer, whereas those on the 9 per cent casein diet were usually dead by the 30th week. Supplementation of the 60 per cent casein diet with vitamin B_{12} and folacin[2] hastened the induction of mammary tumors (group 10). This supplement also appeared to intensify the toxicity of the carcinogen, since it reduced the average survival period from 42 weeks to 32 weeks.

Supplementing the 12 per cent casein diet with desoxyribonucleic acid or with yeast nucleic acid (ribonucleic acid) did not influence the tumor incidence or induction time (groups 7 and 8).

The influence of high protein intake on mammary tumor induction is emphasized when the results with the 40 and 60 per cent casein diets are compared to the results obtained when the casein ranged between 9 and 27 per cent of the diet. The over-all incidence of mammary tumors in animals

TABLE 1

INFLUENCE OF DIETARY CASEIN LEVEL ON TUMOR INDUCTION BY 2-ACETYLAMINOFLUORENE (AAF)

Exp. no.	Group no.	Dietary casein level (per cent)	Av. body weight Initial (gm.)	Av. body weight 16 wks. (gm.)	Av. daily intake per rat Feed (gm.)	Av. daily intake per rat AAF (gm.)	No. rats	No. rats with tumors Mammary	Ear duct	Liver	None	Av. survival (wks.)
	1	9	52	173	6.6	1.98	17	14(22)*	12(25)*	10	0	27
	2	20	51	197	7.0	2.10	8	7(21)	5(25)	4	0	28
1	3	27	49	210	7.0	2.10	4	3(21)	2(24)	3	0	30
	4	40	49	196	7.0	2.10	8	2(25)	3(28)	4	0	32
	5	60	53	168	6.5	1.95	9	0	2(37)	4	3	40†
	6	12	48	198	7.2	2.16	18	16(19)	12(24)	8	0	27
2	7	12‡	56	216	7.6	2.28	6	6(20)	3(25)	2	0	28
	8	12§	57	220	7.6	2.28	6	6(21)	4(24)	3	0	27
	9	60	47	222	7.7	2.31	6	5(23)	4(31)	4	0	42
3	10	60#	46	216	8.0	2.40	12	10(18)	7(23)	4	0	32
	11	9	47	162	6.2	1.86	7	5(21)	4(24)	5	0	28
4	12‖	60	48	184	6.4	1.92	8	1(31)	3(31)	2	3	32**
	13	60	46	214	7.2	2.16	8	5(25)	6(29)	4	0	32**

* The numbers in parentheses are the average tumor induction periods in weeks.

† All these animals were still alive after the 40-week experimental period. They were sacrificed at that time to determine the liver tumor incidence.

‡ The diet was supplemented with 1 per cent of desoxyribonucleic acid.

§ The diet was supplemented with 5 per cent of yeast nucleic acid.

The diet was supplemented with 30 μg. of vitamin B_{12} and 2 mg. of folacin per kilogram.

‖ Rats of this group were pair-fed with those of group 11.

** All these animals were still alive after the 32-week experimental period. They were sacrificed at that time to determine the incidence of liver tumors.

[2] The folacin used in this study was donated by the Lederle Laboratories, Pearl River, New York, and all other vitamins were donated by Merck & Co., Inc., Rahway, N.J.

receiving the diets containing 9–27 per cent of protein was 86 per cent (57 out of 66), contrasted with an incidence of 45 per cent (23 out of 51) for the animals on the high casein diets. The high casein diet, combined with restricted feed intake (groups 4, 5, and 12), resulted in only three mammary tumors in 25 rats (12 per cent incidence), contrasted with an incidence of 80 per cent (nineteen of 24 rats, groups 1 and 11) with a comparable feed consumption of the 9 per cent casein diet.

There is suggestive evidence that the incidence of ear duct tumors may also be influenced. For example, the rats on the high casein diets had a 49 per cent incidence of this tumor, contrasted with an incidence of 63 per cent in the remainder. Moreover, the induction period for ear duct tumors was considerably longer in rats fed the high protein diets than in rats consuming similar amounts of the low protein diets.

Likewise, there is suggestive evidence of a protective effect of high protein diets on liver tumor induction. For example, on equalized feed intakes, five of seven animals in group 11 (9 per cent casein diet) had liver tumors in 28 weeks, whereas only two of eight in group 12 (60 per cent casein diet) had liver tumors in 32 weeks. However, the influence of dietary casein level on liver tumor induction could not be determined accurately in these studies, since many of the animals were kept on experiment to determine the influence of dietary protein level on survival.

DISCUSSION

It is possible that the protective effect of protein observed in the present study is mediated through the liver, and at least suggestive evidence was obtained to indicate that there was less liver damage on the high protein diets than on the low protein diets. Gutmann and associates (6) have recently shown that the extent of disappearance of diazotizable material after the administration of 2-acetylaminofluorene in rats is dependent upon the amount of active liver tissue present. The role of dietary protein in carcinogenesis has received a great deal of attention, and excellent reviews are available (13, 14). It is generally agreed that the most striking protective effect of protein is on tumors originating in the liver, which are induced by the feeding of azo dyes (8, 9, 12).

The present results cannot be explained on the basis of the dietary protein functioning as a detoxifying agent for 2-acetylaminofluorene. At least, if this were the explanation, it would seem that high protein diets would have permitted better weight gains than the low protein diets, feed intake being equal. Diets containing 12–27 per cent of casein were not protective against mammary tumor induction. They nevertheless promoted growth at least as good as that obtained with diets containing 60 per cent of casein.

Since the mammary tumor-inhibiting effects of high protein diets could be largely overcome when a liberal amount of feed was consumed (viz., a higher intake of carcinogen), certain predictions might be made relating level of carcinogen intake to carcinogenicity. In every case, when the food intake exceeded an average of 7.2 gm/rat daily (carcinogen intake of 2.16 mg. or more daily), the protective effect of high protein diets was largely overcome. When the food intake was restricted so that the daily intake of the carcinogen was limited to between 1.80 and 2.10 mg/rat daily, then a protective effect of high dietary casein could be demonstrated. From these and earlier studies (4, 5), it can be further calculated (based on individual food consumption records) that a daily carcinogen intake below 1.80 mg. will result in a low incidence of mammary tumors, even on the 9 per cent casein diet. At this low level of carcinogen intake, however, the reduced incidence of mammary tumors is probably caused at least in part by the low caloric intake, as pointed out earlier (4).

These evaluations serve again to emphasize the importance of controlling the levels of food and carcinogen intake when effects of dietary factors are to be tested. In this connection, it should be mentioned that the protective influence of the high casein diets may be due not to the protein itself but rather to the decreased concentration of sucrose in such diets.

Vitamin B_{12} and folacin had a very definite effect in hastening the carcinogenic process, an effect that can be explained on the basis of a high feed and high carcinogen intake. This supplement promoted earlier tumor development and almost completely eliminated the beneficial effects of high protein intake on survival. Vitamin B_{12} has been reported to enhance the carcinogenic effect of 4-dimethylaminoazobenezene (1), and it was noted in early studies that folacin (teropterin) hastened the induction of mammary tumors in rats fed 2-acetylaminofluorene (3). The growth of a transplantable mammary adenocarcinoma was, however, inhibited more effectively when vitamin B_{12} or folacin was administered with 8-azaguanine than when the latter was administered alone (11).

SUMMARY

When weanling rats consumed a partially purified diet containing 9 to 27 per cent of casein, 57 out of 66 developed mammary tumors (86 per cent incidence). The daily intake of 2-acetylaminofluo-

rene ranged between 1.8 and 2.1 mg/rat. On a corresponding intake of the carcinogen, diets containing 40 or 60 per cent of casein produced a marked reduction in mammary tumor incidence (three of 25 rats or 12 per cent incidence). Increased intakes of food and carcinogen exceeding 2.1 mg. daily largely overcame the protective effects of the high protein diets and resulted in a high incidence of mammary tumors (20 of 26 rats or 77 per cent incidence). Irrespective of level of carcinogen and feed intake, the high protein diets promoted generally improved well-being and prolonged the survival period from an average of 28 weeks to over 40 weeks.

Suggestive evidence was obtained indicating that high casein diets were also partially protective against the induction of ear duct and liver tumors.

Desoxyribonucleic acid or yeast nucleic acid, added to a 12 per cent casein diet, had no influence on tumor induction. The supplementation of a 60 per cent casein diet with vitamin B_{12} and folacin decreased the average survival period from 42 weeks to 32 weeks.

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Heterotransplantation of Human Cancer*

I. Irradiated Rats

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The experiments of Toolan (6) indicated that it was feasible to transplant human neoplasms subcutaneously into rats or mice. Resistance to the foreign tissues was reduced by total-body x-radiation.

Our employment of this method, with the use of 75 different human cancers, resulted in survival of 53 per cent among the transplantations of the first generation. This unanticipated success provided the basis for hope that this technic could be further improved so as to become of practical value.

METHODS

Rats of Slonaker or Wistar strains, of either sex, approximately 1½ months old and weighing between 90 and 150 gm., were employed. Beginning 6–9 days prior to tumor implantation, rats were exposed to total-body irradiation with x-ray, three doses of 200 r being administered on alternate days. X-radiation factors were 200 kvp, 2 mm. Cu, 10 m. amp., t.s.d. 60 cm., and exposure time approximately 4 minutes. No evidence of radiation sickness was noted.

For the implantation of human cancers, neoplastic tissue was obtained as fresh as possible from operating or autopsy room, or surgical pathology laboratory. It was occasionally found that, even after refrigeration of the tumors for 48 hours, success with transplantation was achieved. However, in general, fresher specimens of tissue were preferred.

The tissue was not received in sterile condition. It was attempted to inhibit bacterial growth by suspending the minced cancer tissue, 0.5 cc., in an equal volume of penicillin solution containing 5,000 units/cc, during preparations for implantation.

For transplantation, rats were anesthetized with ether. Fragments of tumor approximately 2 mm. in diameter, which did not appear grossly necrotic or composed mainly of inflammatory tissue or stroma, were chosen. Tumors were implanted subcutaneously by means of a trocar or fine forceps. Usually two different sites were used for simultaneous implantation in the same animal. From four to six rats were employed for each tumor. No mortality attended the transplantation procedure.

Nodules were removed 7–10 days after implantation. At this time they varied in size from 2-mm. pieces, as originally implanted, up to 1 cm. in diameter. The average diameter was 5 mm. or less. All nodules of tumor were submitted for microscopic examination, except when further implantations were attempted. Daily examinations for nodules at injection sites were omitted after this was found to be a poor criterion of growth. Granulomatous inflammatory reactions often interfered with gross diagnosis as to the presence or the dimensions of the tumors.

The most successful interval for harvesting tumors was found to be about 8 days. Generally, after about 10 days the transplants appeared completely absorbed grossly and microscopically. However, an occasional tumor would remain viable as a 5-mm. nodule for a month or more, with microscopic evidence of active proliferation shown by mitoses.

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RESULTS

The rats did not show any generalized body disturbances or gross anatomic changes following human cancer transplantations.

The 75 human cancers and the results of their transplantation are listed in Table 1. Each positive instance, defined as the growth or survival of living cancer tissue, was confirmed by microscopic identification of human cancer tissue from the rat. When there were too few cancer cells to produce structural patterns recognizable as malignant growths, the transplants were considered questionable, although a few individual cancer cells might be present. Negative results meant either necrosis and death of all cancer tissue identified, or the absence of recognizable cancer cells.

Heterologous growth with invasion of rat tissues by the cancer was not frequent. It appeared doubtful that the transplant was often vascularized by the host tissues. The majority of transplants were living, with evidence of degeneration outweighing signs of growth. Mitoses were found in several transplants. However, it was evident in most instances that human cancers persisted, rather than grew, in the rats.

Because of the relatively small number of various individual tumors included, no general conclusions could be drawn. From the results, there appears to be no reason to anticipate failure in any type of human cancer transplant, up to this time. Differences in single instances seemed to have been

influenced by the condition of the piece of tumor chosen, as much as by any single factor.

In a number of rats, microscopic examination revealed human stromal tissue well preserved but without cancer cells. In this regard, it should be emphasized that normal tissue structures, such as human salivary gland ducts, retained their normal appearance in transplants (5).

Transplants into a second generation were attempted in ten cases, with success in five (Table 2). Two of these five were transplants from the rat into the hamster cheek pouch. All five positive instances did indicate both grossly and microscopically heterologous proliferative growth of human cancer. It was felt preferable to determine microscopically the survival of the first-generation transplants at the expense of saving sufficient tissue for further transplantation, so that only a few second-generation implants were attempted.

One group of rats was irradiated with only 180 r, owing to errors of calibration. The results are listed in Table 3. Glandular carcinomas were transplanted with less success than epidermoid carcinomas at this dosage level.

A limited survey of some potential growth-stimulating substances was followed without success. Successful transplantations were made in 38 per cent of the rats given implants of stilbestrol, compared to 45 per cent in untreated controls, with the use of the same 24 tumors. Two of six tumors were successfully transplanted, in animals treated with aureomycin (4), with one out of six successes in untreated control animals.

TABLE 1

RESULTS OF HUMAN CANCER TRANSPLANTS IN IRRADIATED RATS

	Cases	Positive	Negative	Questionable
Adenocarcinoma	22	12	9	1
Large intestine	15	9	5	1
Ovary	2	0	2	0
Stomach	1	0	1	0
Endometrium	1	1	0	0
Breast	1	0	1	0
Prostate	1	1	0	0
Kidney	1	1	0	0
Carcinoma simplex	17	11	4	2
Breast	9	6	2	1
Stomach	5	2	2	1
Ovary	1	1	0	0
Kidney	1	1	0	0
Lung	1	1	0	0
Epidermoid carcinoma	14	7	7	0
Cervix	3	1	2	0
Esophagus	2	0	2	0
Tongue	2	2	0	0
Skin	3	1	2	0
Lip	2	1	1	0
Palate	1	1	0	0
Lung	1	1	0	0
Undifferentiated carcinoma, lung	3	1	1	1
Sarcomas	11	6	5	0
Leiomyo-	3	3	0	0
Fibro-	2	0	2	0
Synovial	1	0	1	0
Osteogenic	1	0	1	0
Lympho-	1	0	1	0
Chondrofibro-	1	1	0	0
Mixed	1	1	0	0
Undifferentiated	1	1	0	0
Gliomas	2	0	1	1
Glioblastoma	1	0	1	0
Ependymoma	1	0	0	1
Melanoma	2	0	2	0
Neuroblastoma	1	1	0	0
Undifferentiated neoplasm, brain	1	0	1	0
Tumors of doubtful malignancy	2	2	0	0
Leiomyoma	1	1	0	0
Meningioma	1	1	0	0
Totals	75	40	30	5

TABLE 2

SECOND-GENERATION CANCER TRANSPLANTS

	Cases	Positive	Negative	Questionable
Epidermoid carcinoma	3	2	1	0
Carcinoma simplex*	1	1	0	0
Adenocarcinoma*	1	0	1	0
Leiomyosarcoma	2	1	0	1
Fibrosarcoma*	1	1	0	0
Undifferentiated cancers	2	0	1	1
Totals	10	5	3	2

* Hamster cheek pouch used for second transplants.

TABLE 3

RESULTS OF CANCER TRANSPLANTS AFTER 180 r

	Cases	Positive	Negative
Epidermoid carcinoma	9	4	5
Adenocarcinoma	10	1	9
Carcinoma simplex	7	0	7
Undifferentiated carcinoma, lung	1	0	1
Melanoma	3	2	1
Sarcomas	4	1	3
Chondro-	1	1	0
Lympho-, reticulum-cell	2	0	2
Retroperitoneal	1	0	1
Glioblastoma	1	0	1
Totals	35	8	27

DISCUSSION

Heterologous cancer transplantation in the irradiated rat is as yet of little practical value. The proportion of successful takes and the evidence of

growth in the transplants are not sufficiently satisfactory for routine use. However, in our laboratory the results compare favorably with those of others (1, 3) who used the anterior eye chamber, as to the number of animals required, and time, skill, and experience necessary for success. Vigorous growth of transplanted cancers comparable to that observed by Greene (2) has not been found in this series of irradiated rats.

The method of Toolan is recommended as useful and as offering an opportunity for studying human cancer in an animal host.

SUMMARY

Forty of 75 human cancers (53 per cent) survived transplantation to irradiated rats. Five of ten second-generation implants actually grew, and proliferative enlargements were subsequently identified microscopically. Little evidence was found for active growth in most persisting first-generation heterologous transplants. The technic described is considered sufficiently successful to warrant its wider use.

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Heterotransplantation of Human Cancer

II. Hamster Cheek Pouch*

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A technic developed by Lutz, Handler, and associates (1–4) employed the cheek pouch of the hamster (*Mesocricetus auratus*) as a site for transplantation of tissues, including animal and human cancers. Through the courtesy of these workers, we learned their method and employed it in experiments on the heterologous transplantation of human cancers.

Sixty-five human neoplasms were so implanted, with successful growth in 30 (46 per cent).

METHODS

Hamsters of either sex, 1½–3 months old and weighing 40–100 gm., were used.

Two technics of transplantation were used—that of Lutz (4) and a modification—with comparable results.

For stretching the everted cheek pouch, a simple animal holder was used as originally described by Lutz. In the modified technic, the hamster was anesthetized with ether and the cheek pouch simply everted over one finger. A fragment of human tumor, selected and treated as described in the preceding communication, was implanted beneath the mucosa with a No. 18 trocar. Care was taken to avoid extrusion of the fragment through the wound of introduction, by working it away from this site. Refrigeration of the tumor for 48 hours did not appreciably alter its viability.

After implantation the hamster was observed for tumor growth by everting the cheek pouch approximately every 4 days. When the nodule showed peripheral hemorrhage or edema, its vascularity decreased, or it became softened, the tissue was removed for histologic examination or further transplantation.

Usually the tumor was implanted in one pouch of each of six hamsters. Occasionally, after negative results, the same pouches were used for further transplants.

RESULTS

In general, after about 4 days an enlarged nodule was evident beneath the cheek pouch mucosa. It was gray-pink, spherical, and smooth-surfaced. Vascularization was not always evident at this time. With certain tumors, the nodules became softened, shrank, and were generally absorbed within 10–20 days. No generalized effects on the hamsters were observed.

Measurement of changes in the size of the nodules was not successful as an index of cancer growth. Peripherally, granuloma formation closely simulated cancer, and it was not possible grossly to distinguish accurately the margin between implanted tumors and reactive host tissues.

In Table 1 are listed the results of 65 transplants of human cancer. "Positive" results refer to survival or growth of cancer, "negative" to complete degeneration or absence of cancer, and "questionable" to presence of a few doubtful cancer cells. As noted in irradiated rats, there is not sufficient evidence as yet to predict whether any particular type of neoplasm would or would not grow heterologously. Fortunate or unfortunate choice of the fragment for implantation was the one factor of most importance.

The survival of all implants was confirmed by microscopic examination. Vascularization was more prominent grossly than microscopically, and the margin of cancer tissue often appeared confined histologically by leukocytic infiltration. The hamster responded by exuberant histiocytic granulomatous response to the foreign tissue. The cancer cells appeared healthy, with or lacking mitoses, although signs of degeneration were occasionally present. No changes in growth characteristics or type of cancer were noted.

Second-generation transplantation was attempted, employing twenty neoplasms (Table 2). As with animal neoplasms (5), the relative proportion of successes was increased. Third-generation transplants succeeded with one of three carcinoma simplex tissues and two of two epidermoid carcinomas tested and failed with one leiomyosarcoma. In these positive implants, proliferative growth rather than mere persistence was observed.

In another series, twelve hamsters were irradi-

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ated with 300–600 r total body x-ray doses before implantation with twelve human neoplasms. The irradiation factors were 200 kvp, 2 mm. Cu, 10 m. amp., t.s.d. 36.5, and 60 cm. with exposure times 1–4 minutes, according to dose. Fifty-eight per cent of these tumors were successfully grown in both the irradiated and unirradiated control animals. Transplantation of thirteen neoplasms was successful in hamsters treated with stilbestrol and in untreated control hamsters (15 per cent), and with four neoplasms in hamsters given aureomycin and in their untreated controls (75 per cent). No evidence of more favorable effects was obtained from these additional procedures.

TABLE 1

HAMSTER CHEEK POUCH TRANSPLANTS OF HUMAN CANCERS

	Cases	Positive	Negative	Questionable
Adenocarcinoma	14	6	7	1
Endometrium	4	0	3	1
Large intestine	3	2	1	0
Prostate	2	1	1	0
Salivary gland	2	1	1	0
Ovary	1	0	1	0
Kidney	1	1	0	0
Thyroid	1	1	0	0
Carcinoma simplex	12	5	7	0
Breast	5	2	3	0
Stomach	4	2	2	0
Ovary	1	1	0	0
Kidney	1	0	1	0
Endometrium	1	0	1	0
Epidermoid carcinoma	18	9	9	0
Lung	3	2	1	0
Cervix	3	1	2	0
Skin	3	1	2	0
Lip	2	1	1	0
Tongue	2	2	0	0
Larynx	2	2	0	0
Anus	1	0	1	0
Palate	1	0	1	0
Esophagus	1	0	1	0
Adenoacanthoma, uterus	1	1	0	0
Renal-cell carcinoma	1	0	1	0
Undifferentiated carcinoma, lung	2	1	1	0
Sarcomas	10	7	2	1
Leiomyo-	2	2	0	0
Undifferentiated	2	0	2	0
Chondrofibro-	1	1	0	0
Fibro-	2	1	0	1
Synovial	1	1	0	0
Osteogenic	1	1	0	0
Mixed	1	1	0	0
Melanoma	2	0	2	0
Neuroblastoma	1	0	0	1
Brain Tumors	3	1	2	0
Glioblastoma	1	0	1	0
Ependymoma	1	0	1	0
Craniopharyngioma	1	1	0	0
Undifferentiated malignant tumor from brain	1	0	1	0
Totals	65	30	32	3

TABLE 2

SECOND-GENERATION HAMSTER CHEEK POUCH CANCER TRANSPLANTS

	Cases	Positive	Negative	Questionable
Adenocarcinoma*	1	0	1	0
Carcinoma simplex	4	2	2	0
Carcinoma simplex*	1	1	0	0
Epidermoid carcinoma	6	3	3	0
Epidermoid carcinoma*	1	1	0	0
Adenoacanthoma	1	1	0	0
Undifferentiated carcinoma of lung	1	0	0	1
Craniopharyngioma	1	1	0	0
Sarcoma	4	2	2	0
Leiomyo-	2	1	1	0
Fibro-*	1	1	0	0
Chondrofibro-	1	0	1	0
Totals	20	11	8	1

* Tumors obtained after first implantations subcutaneously in irradiated rats.

DISCUSSION

In our experience, employment of hamster cheek pouches for heterologous cancer implants of human origin has appeared to be of great potential value. It has the advantages of ease of implantation and of observation. Vascularization may be studied by reflected or transmitted light, and the tumor may be easily photographed. Changes in tumor consistency and color are readily observed beneath the thin mucosa.

With experience in the proper choice of tissue fragments, an increased proportion of successes is anticipated, comparable to the results of the originators of this method. Furthermore, this makes possible the employment of the gross measurement of tumor implants as an index of their growth.

The technic has developed to a point where intensive study of selected types of cancer is now feasible. Further extension to different neoplasms is also contemplated.

SUMMARY

In the hamster cheek pouch, 30 of 65 human neoplasms persisted after heterologous transplantation (46 per cent). Second-generation transplantations were successful with eleven of twenty cancers, and third-generation growth in three of six cases. The method is relatively simple and appears

to be of great potential value in growth studies of human cancer tissue. Treating host animals with stilbestrol or aureomycin or x-ray did not affect transplant survival.

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Heterotransplantation of Human Cancer

III. Chorioallantoic Membranes of Embryonated Eggs*

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The chick embryo has been used successfully for the culture of bacteria (5), viruses (4), normal (3) and neoplastic tissues (1, 2, 6). Its extensive employment for heterologous transplantation of human cancers has not been reported.

METHODS

Embryonated chicken eggs incubated from 5 to 12 days were employed. Before and after implantation the eggs were incubated at 37° C. and about 80 per cent humidity. The eggs were turned over twice a day for the first 3–4 days and daily thereafter until injection. Somewhat better results were obtained with eggs about 6–7 days old, since fewer embryos were killed by the transplantation procedure. The "false air sac" method adapted from virus culture (4) was used.

After 6 days of incubation, the positions of the embryo and air sac were located by candling and traced with pencil on the shell. Some eggs were then x-radiated with specified doses and kept 24 hours longer in the incubator before injection of tumor. Either three daily doses of 25–75 r or single exposures to 150–250 r, 1 day before injection, were employed. Radiation data were the same as given in the preceding communications.

The outlined pencilled areas were painted with merthiolate, and the carborundum disc of an electric cutting tool was used to cut a small window approximately 1 cm. square just beside the embryo. A small puncture was then made into the air sac to lower the embryo from the shell and to prevent its being mechanically damaged. The shell and shell membrane were removed with sterile forceps. A sterile 1-cc. syringe and 20-gauge 1½-inch needle were used to inject the tumor directly into the chorioallantoic membrane. For accurate localization of the implant later, it was found desirable to introduce small amounts of carbon black with the tumor. Cellulose tape was used to close the window, as it was considered sterile and permitted easy observation of the implants after injection.

Intra-yolk-sac injections (6) were also attempted but were not successful.

Choice of a viable portion of tumor was of the greatest importance. Since the tumor was not received in a sterile condition, it was first treated by suspending minced fragments in a penicillin solution containing 5,000 units in 1 cc. aqueous solution, for 1–4 hours before inoculation. From two to twelve eggs were inoculated with each tumor. A few cancers were first transplanted into irradiated rats or hamster cheek pouches and secondary implants later attempted into eggs.

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After 1–8 days' further incubation, the eggs were allowed to cool in the refrigerator. The shells were cracked. The transplants were located, fixed in Zenker-formol, imbedded in paraffin, cut, and stained with hematoxylin and eosin for microscopic examination.

RESULTS

Tabulation of the transplants is shown in Table 1, with successful persistence or growth in 28 of the 59 different human cancers employed (47 per cent). "Positive" results mean persistence, or, rarely, growth of human cancers; "negative" refers to absence of any demonstrable living cancer cells; and "questionable" means identification of a few possibly viable cancer cells. A total of 375 normal embryonated eggs were employed. Of the 149 surviving until harvesting, 36 were positive (24 per cent) and 26 questionable (17 per cent). Of 245 irradiated egg embryos, 178 were harvested, 38 were positive (21 per cent), and 30 questionable (17 per cent). Thus, the proportion of over-all takes was relatively small, with or without irradiation, compared to the total number of eggs used.

Indications of growth of the transplants were rarely observed, and vascularization was not evident grossly. As a rule, persistence or survival of tumor, rather than growth, better described the microscopic appearance of the human cancers. In general, chicken leukocytes surrounded the human tissue, at times with a granulomatous response. Mitoses in tumors were observed occasionally, but degenerative changes were much more frequent.

Transplantation was not attempted beyond the first generation, except for one second transplant of epidermoid carcinoma of the tongue, which was identified in one of the five eggs employed.

As with the other methods reported, sarcomas appeared to be transplanted most easily, since, among 47 eggs employed, thirteen were positive for sarcoma and six questionable. Adenocarcinomas and carcinoma simplex survived in only 32 of 146 eggs, with 35 doubtful or degenerated cancers. Epidermoid carcinoma was found in fifteen of the 99 eggs used, besides three doubtful cases. The

successful transplant yield was about one-quarter of the eggs with sarcomas, one-fifth with glandular carcinomas, and about one-seventh with epidermoid carcinomas. The best proportion of persistence of any tumor occurred in Hodgkin's disease, with which eighteen eggs were injected. Ten were positive, three degenerated, and five negative.

Transplants of two human cancers first to hamster cheek pouch and thence to eggs succeeded with a leiomyosarcoma and failed with an endometrial adenoacanthoma. Transplants of two other human cancers were made into irradiated rats and later injected into eggs. Both failed to survive.

Sixty-eight eggs were injected with nontumorous tissue. Cystic teratoma (dermoid cyst) of ovary, cholesteatoma of middle ear, inflamed lymph node, nodular goiter, chronic dermatitis, and radiation-damaged bladder specimens all persisted in egg implants.

TABLE 1

HUMAN CANCER TRANSPLANTS ON CHORIOALLANTOIC MEMBRANES OF CHICK EMBRYOS

	Cases	Positive	Negative	Questionable or degenerated
Adenocarcinoma	15	4	4	7
Large intestine	4	1	0	3
Prostate	3	0	1	2
Abdominal wall	1	1	0	0
Cervix	1	0	1	0
Endometrium	2	0	2	0
Chest wall	1	1	0	0
Gall bladder	1	1	0	0
Perineum	1	0	0	1
Thyroid	1	0	0	1
Carcinoma simplex	14	7	3	4
Breast	10	5	1	4
Ovary	2	1	1	0
Endometrium	1	0	1	0
Stomach	1	1	0	0
Undifferentiated carcinoma	3	1	0	2
Lung	2	1	0	1
Ovary	1	0	0	1
Renal cell carcinoma	2	1	1	0
Epidermoid carcinoma	11	5	4	2
Tongue	3	0	2	1
Esophagus	3	2	1	0
Lung	1	1	0	0
Palate	1	0	1	0
Mouth	1	1	0	0
Cervix	2	1	0	1
Sarcoma	6	6	0	0
Leiomyo-	1	1	0	0
Fibro-	1	1	0	0
Fibrolipo-	1	1	0	0
Mixed	1	1	0	0
Kaposi's	1	1	0	0
Undifferentiated	1	1	0	0
Miscellaneous	10	4	4	2
Melanoma	1	0	1	0
Hodgkin's disease	2	2	0	0
Adenoacanthoma, endometrium	2	0	2	0
Anaplastic carcinoma, thyroid	1	0	0	1
Malignant adenoma, rectum	1	0	1	0
Neuroblastoma, lung	1	1	0	0
Mixed tumor, salivary gland	1	1	0	0
Craniopharyngioma	1	0	0	1
Totals	61*	30	14	17

* Discrepancy with text due to one second generation transplant and one tumor first transplanted into hamster cheek pouch.

DISCUSSION

The technic of employing the chorioallantoic membrane is relatively difficult, time-consuming, and requires meticulous attention to sterile technic, as well as considerable practice for success. With these factors under control, however, it offers about the same percentage of successful human cancer transplantation as the other methods employed in our laboratory. By success is meant persistence of surviving cancer; gross or microscopic proliferative growth is uncommon in eggs. Bacteriologic controls similar to those used in operating rooms must be employed.

After successful heterotransplantation in other media, future egg implants may prove more frequently successful. As in homologous animal tumor transplantations, it appears to be the first generation growth which is the most difficult to obtain. Once better tissue adaptation is achieved, transplantation is facilitated.

No explanation for the easier transplantability of sarcoma tissues is advanced, but perhaps this is due to a superior metabolic adaptability of sarcomas to the anerobic conditions characteristic of embryonic growth.

X-radiation of the embryos does not appear to increase the proportion of tumor survivals. Isotopes may be more useful in this connection. A search for growth-stimulating factors is also necessary. However, the fatality rate of embryos increases proportionately to the number of injections made. Perhaps isotopes or growth stimulants may best be injected at the same time that the tumor implant is done.

SUMMARY

Twenty-eight of 59 different human cancers (47 per cent) successfully survived transplantation to the chorioallantoic membranes of chicken egg embryos. A total of 620 incubated eggs were used. In terms of the 327 eggs whose embryo survived implantation, the positive harvest was 23 per cent, with or without prior x-radiation. Sarcomas sur-

vived in about one-quarter of the eggs harvested, glandular carcinomas in one-fifth, and epidermoid carcinomas in one-seventh.

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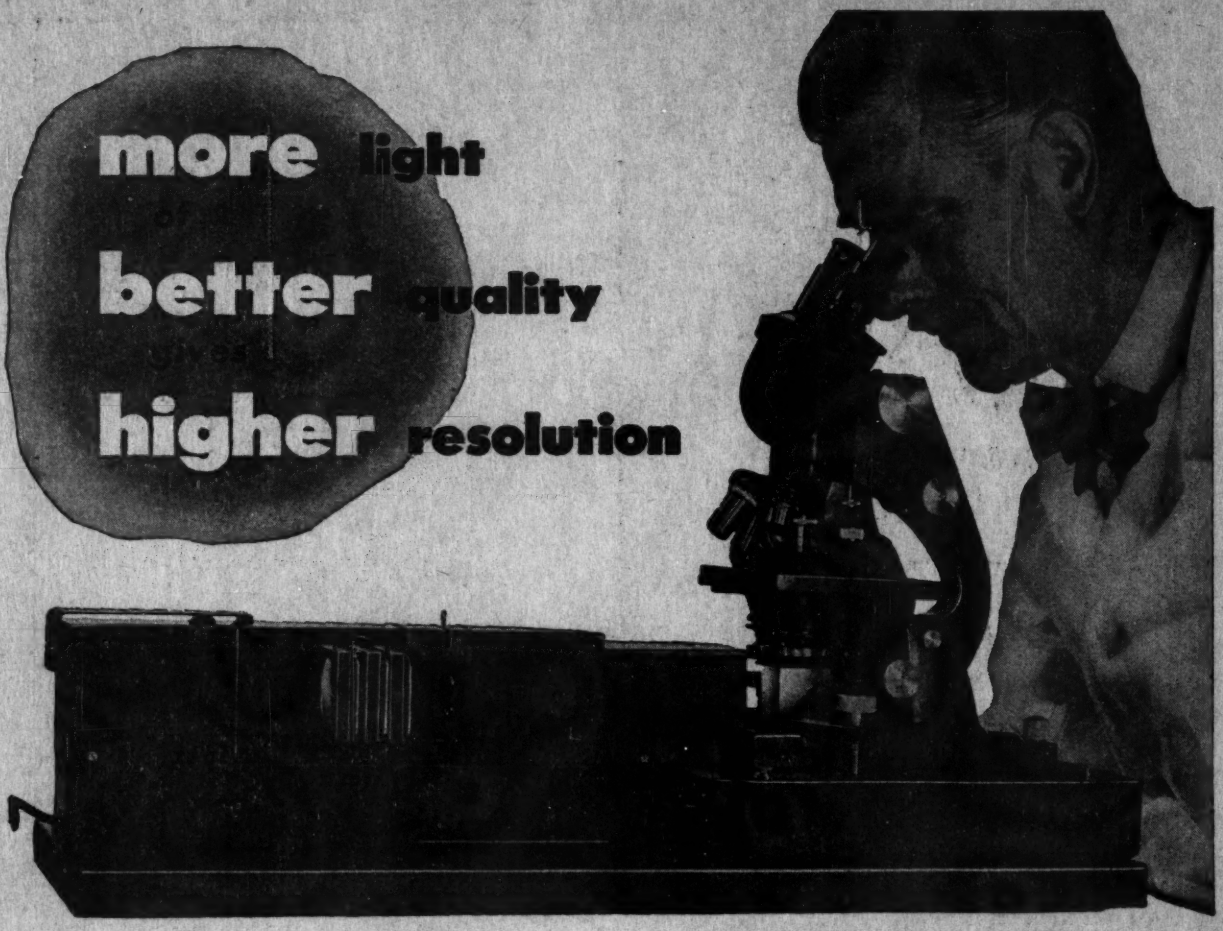

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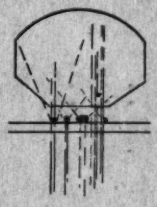

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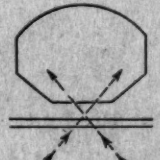

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